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THE EFFECTS OF DIETS LOW IN CHOLINE UPON LIVER FUNCTION, GROWTH, AND DISTRIBUTION OF FAT IN THE WHITE RAT.

D. L. MACLEAN, J. H. RIDOUT, AND C. H. BEST.
The School of Hygiene, University of Toronto, Toronto.

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THE purpose of these experiments was to make further studies of the effects of diets low in choline and other lipotropic factors on liver function, on the rate of increase in weight, and on the distribution of fat in the white rat. These experiments were conducted over a much longer period than in our previous studies. The results show that while there is definite interference with certain functions of the liver as judged by its ability to excrete dye and to store glycogen, these properties are retained to a surprising extent by the extremely fatty liver tissue. Some preliminary observations on the excretion of ketone bodies by groups of rats whose livers contained different amounts of fats are also recorded. The results of the studies on changes in weight and distribution of fat confirm and extend those which have been reported previously.

METHODS.

White rats of the Wistar strain were placed in individual cages and provided with a diet made up of the following constituents: The protein (10 p.c. by weight) was provided in the form of a meat powder. This powder was prepared from beef muscle after thorough extraction of the minced tissue with strong ethyl alcohol. The residue after the alcoholic extractions was dried in a vacuum oven and ground to a fine powder. This material had previously been shown to possess less lipotropic activity than the so-called "fat- and vitamin-free" casein. The diet also contained 40 p.c. beef dripping, 43 p.c. commercial sucrose, 5 p.c. salt mixture, 2 p.c. agar and vitamins A, D and B_1. Each animal received four international units of vitamin B_1 and a cod-liver oil concentrate which provided 75 units of A and 8 units of vitamin D daily.

The bromsulphalein test of liver function was conducted in the following manner: A 2 p.c. aqueous solution of the dye was injected into the jugular vein. Exactly fifteen minutes later the animals were bled and the serum secured. One drop of a 20 p.c. KOH solution was added to the serum of each rat and the presence or absence of the dye colour noted. The amount of the dye solution injected varied with the weight of the animals. Those weighing

from 140 to 174 g. received 0·12 c.c. ; from 175 to 239 g., 0·18 c.c. ; and from 240 to 270 g., 0·25 c.c. A tuberculin syringe graduated in hundredths of a c.c. was used for the injections. Normal rats, in our experience, never retain dye in their serum under the conditions of this test. In one series of experiments (Table V) the dose of the dye was slightly greater than that described above, but the amount used did not give a positive result on normal rats of the same weight.

For the glycogen storage test the animals were fasted during the previous twenty-four hours. In the series of experiments described in Tables IIIc and IV, each rat was given approximately 2·0 g. of dextrose as a 50 p.c. aqueous solution by means of a small stomach-tube. At the end of three hours the rat was anæsthetized with amytal. The abdominal cavity was opened and the liver was rapidly removed and transferred to a tared centrifuge cup containing boiling potash solution. Ligatures were placed around the upper and lower end of the intestinal tract. The stomach and small and large intestines were carefully taken out and placed in a beaker with distilled water as the first stage in the estimation of reducing substances. The exact amount of dextrose which was administered and the amount of reducing substance which remained in the gastro-intestinal tract at the end of three hours were determined by the method of Cori (1926).

In the control animals a similar volume of water was given in place of the dextrose solution. The amount of reducing substance present in the gastro-intestinal tract was estimated, and this amount was subtracted from that found in the experimental series. This latter value was then subtracted from the total amount of dextrose which was given and the quantity absorbed during the three-hour period was thus estimated. The absorption coefficient is based on the amount of dextrose absorbed by a 100-g. rat in one hour.

The liver glycogen was estimated by a modification of Pflüger's method. The average liver glycogen for the control rats was subtracted from the value found for the experimental animals. The supernatant liquid and washings from the glycogen precipitate were collected, and after further saponification, fat estimations were made in the usual manner. The term " body-fat " used in this paper refers to that of the carcase minus that of the intestinal tract and the liver.

In the results reported in Table IIIa and b, the technique used was the same as that described with the exception that 600 mg. of dextrose were given per 100-g. rat, and the deposition of liver glycogen was determined after a four-hour absorption period.

Reducing substances were estimated by the DeLong modification of the Shaffer-Hartmann method (1927). The excretion of ketone bodies was determined as previously described by Best and Campbell (1936).

EXPERIMENTAL RESULTS AND COMMENTS.

One hundred and seventy rats were used in the first experiment. These were divided into two groups. All the animals received the same amount of the diet. One-half of them were given choline. The first observations were made after 22 days. Sixteen of the animals receiving no choline and 17 of the

series receiving choline were killed to determine the liver-fat. The bromsulphalein test was conducted on all these animals, but was found to be negative in every case (Table IA). The next group of rats were examined 36 days after the beginning of the experiment. The liver-fat had risen in the animals receiving no choline to 25·8 p.c. while it remained at 5·3 p.c. in those which had received it. The bromsulphalein test was performed on all these animals, and was found to be positive in 8 of those which had received no choline and negative in 9. The test was negative throughout in the 16 rats which received choline (Table IB). The third series were examined after 50 days. These results are given in detail in Table II. There was a surprisingly uniform deposition of fat in the animals which received no choline. There was an insignificant rise in the fat content of the livers of the animals which received choline. The bromsulphalein test was conducted on all the animals, and was found to be positive in 7 cases in the animals which received no choline and negative in 10. It was negative throughout in the 17 animals which received choline.

In Table IIIA the results of observations made 54 days after the start of the experiment are summarized. The glycogen storage test was conducted upon these animals. In the 11 which received no choline in the diet the total liver glycogen averaged 110 mg., while in a similar group which received choline the average liver glycogen was 165 mg. To 6 animals of each series water was given instead of sugar solution. In these groups the average deposition of glycogen in the liver was 5 mg. This amount was subtracted from the total found in the experimental animals to give the figures reported in the tables.

After 62 days the glycogen storage test was performed on 15 rats of each group. The average total glycogen of the liver was 80 mg. in the animals which received no choline, and 110 mg. in those to which choline was administered (Table IIIB).

In the next experiment 96 rats were studied. The same diet was used and approximately the same amount of choline was provided. The first

TABLE I.

	Number of rats.	Average change in weight (g.).	Liver-fat. Total (g.).	Liver-fat. p.c.	Body-fat. Total (g.).	Body-fat. p.c.	Bromsulphalein retention.	Duration of experiment (days).
No choline (a).								
A	16	+1	1·52	18·2	14·2	9·2	Negative throughout.	22
B	17	+1	2·89	25·8	14·6	9·2	Positive 8 cases; negative 9 cases.	36
With choline (b).								
A	17	+7	0·30	4·6	14·9	9·2	Negative throughout.	22
B	16	+12	0·41	5·3	22·0	12·2	Ditto.	36

Remarks.—Average daily food intake 9·5 g. Each rat in (b) group received 33 mg. choline daily.

TABLE II.

Rat No.	Change in weight (g.).	Liver-fat. Total (g.).	Liver-fat. p.c.	Body-fat. Total (g.).	Body-fat. p.c.	Bromsulphalein retention.
No choline (a).						
35	−8	3·13	31·1	12·2	9·4	—
36	−20	3·26	31·0	11·8	9·4	—
37	−30	2·90	31·5	14·0	10·8	—
38	−12	3·06	31·2	14·3	10·6	—
39	−18	4·22	31·6	13·8	11·0	—
40	−10	3·62	35·3	18·3	12·7	+
41	−36	2·84	28·4	14·2	9·9	—
42	+8	3·45	31·6	27·6	16·1	+
43	−8	2·90	29·0	18·2	10·7	+
44	+6	4·90	32·4	16·3	9·8	+
45	+8	3·48	31·6	17·7	11·8	+
46	−20	3·73	32·0	9·4	6·0	—
47	−38	1·49	15·2	14·5	7·4	—
48	−30	3·32	32·3	13·5	9·8	—
49	+2	3·01	32·8	16·3	11·9	—
50	−6	3·76	34·8	11·2	8·2	+
51	−22	4·90	29·9	24·2	10·9	+
Average	−14	3·41	30·7	15·7	10·4	Positive 7 cases; negative 10 cases.
With choline (b).						
120	+22	0·48	5·1	—	—	—
121	+23	0·40	4·7	24·2	12·0	—
122	+50	0·45	5·5	27·4	13·3	—
123	+36	0·39	5·0	25·1	12·8	—
124	+14	0·34	4·7	23·1	13·0	—
125	−14	0·33	3·8	20·9	9·3	—
126	+49	0·63	7·0	33·2	16·2	—
127	+12	0·36	4·6	27·6	12·1	—
128	+8	0·33	4·8	14·9	7·7	—
129	+20	0·27	3·8	25·0	16·0	—
130	+8	0·33	4·3	27·6	13·4	—
131	+10	0·35	4·2	18·5	10·2	—
132	+8	0·26	4·0	21·0	11·4	—
133	+16	0·27	3·8	18·2	13·2	—
134	+6	0·21	4·8	17·9	13·8	—
135	−4	0·24	3·9	13·7	10·4	—
136	+20	0·23	4·0	21·6	14·4	—
Average	+17	0·35	4·6	22·5	12·4	Negative throughout.

Remarks.—Duration of experiment 50 days. Average daily food intake 9·5 g. Each rat in (b) group received 33 mg. choline daily.

TABLE III.

No choline (a).

Number of rats.	Average change in weight (g.).	Liver-fat. Total (g.).	Liver-fat. p.c.	Body-fat. Total (g.).	Body-fat. p.c.	Liver glycogen. Total (g.).	Liver glycogen. p.c.	Duration of experiment (days).
A 11	−14	3·09	28·3	15·6	10·1	0·110	1·1	54
B 15	−3	3·42	28·6	13·4	8·4	0·080	0·7	62
C 13	−10	3·31	27·9	11·8	7·7	0·053	0·4	58

With choline (b).

A 12	+10	0·33	4·9	23·4	13·0	0·165	2·5	54
B 15	+16	0·29	4·3	24·5	13·1	0·110	1·7	62
C 13	+18	0·31	4·5	15·4	8·3	0·087	1·3	58

Remarks.—Average daily food intake, A, 9·8 g.; B, 9·3 g.; C, 10 g. Each rat in (b) group received 33 mg. choline daily (A and B) or 35 mg. (C).

TABLE IV.

No choline (a).

Rat No.	Change in weight (g.).	Liver-fat. Total (g.).	Liver-fat. p.c.	Body-fat. Total (g.).	Body-fat. p.c.	* Abs. coeff. (g.).	Liver glycogen. Total (g.).	Liver glycogen. p.c.
19	−9	3·17	29·6	14·1	9·5	0·23	0·075	0·7
20	−29	4·26	42·8	10·2	8·2	0·23	0·052	0·5
21	−4	2·78	25·0	16·5	10·4	0·19	0·078	0·7
22	+3	4·05	29·6	12·8	7·3	0·22	0·065	0·4
26	+26	3·58	31·6	11·7	8·2	0·26	0·078	0·7
27	−15	3·83	28·4	15·0	9·3	0·18	0·081	0·6
29	−25	1·07	18·2	6·4	6·4	0·24	0·063	1·1
30	−23	3·30	27·5	8·6	6·6	0·21	0·061	0·5
Average	−10	3·26	29·1	11·9	8·3	0·22	0·069	0·6

With choline (b).

67	+32	0·38	5·7	30·9	18·8	0·20*	0·174	2·4
68	+40	0·26	3·5	22·3	12·1	0·21	0·125	1·7
69	+42	0·23	3·7	28·1	15·1	0·14	0·118	1·9
70	+34	0·24	3·5	16·5	9·7	0·22	0·126	1·8
71	+4	0·33	5·0	11·1	7·6	0·20	0·074	1·1
75	−8	0·26	5·7	5·4	5·1	0·21	0·057	1·3
76	+40	0·29	3·7	23·1	10·3	0·20	0·117	1·5
124	+28	..	..	20·4	11·1	0·17	0·102	1·4
125	−8	0·19	3·7	7·2	6·4	0·15	0·068	1·3
Average	+23	0·27	4·3	18·3	10·7	0·19	0·107	1·6

Remarks.—Duration of experiment 70 days. Average daily food intake 10 g. Each rat in (b) group received 35 mg. of choline daily.

* Absorption coefficient is the amount of glucose absorbed per 100 g. rat per hour.

TABLE V.

No choline (a).

Number of rats.	Average change in weight (g.).	Liver-fat. Total (g.).	Liver-fat. p.c.	Body-fat. Total (g.).	Body-fat. p.c.	Bromsulphalein retention.
18	−18	3·20	30·2	11·2	8·0	Positive 8 cases; negative 9 cases.

With choline (b).

17	+8	0·35	4·8	16·6	9·8	Negative throughout.

Remarks.—Duration of experiment 75 days. Average daily food intake 10 g. Each rat in (b) group received 35 mg. choline daily.

TABLE VI.—*Average Daily Ketone Excretion as mg. Acetone per 200 g. Rat during Fasting.*

Group.	Days of fasting. 1. (mg.)	2. (mg.)	3. (mg.)	4. (mg.)	5. (mg.)	6. (mg.)	7. (mg.)
I	0·6	4·2	7·4	2·9	..	..	..
II	1·1	7·5	5·3	2·5	..	..	..
III	1·3	5·7	3·2	1·8	1·1	0·7	0·8

TABLE VII.—*The Ketone Excretion of Rats with Fatty Livers during Fasting.*

Group.	Time on low choline diet (days).	Beginning of fast. Liver-fat. Total (g.).	Liver-fat. p.c.	Body-fat. Total (g.).	Body-fat. p.c.	Length of fast (days).	End of fast. Liver-fat. Total (g.).	Liver-fat. p.c.	Body-fat. Total (g.).	Body-fat. p.c.	Total ketone excretion during 4-day fast as mg. acetone per 200-g. rat.
I	21	1·53	14·9	26·5	12·8	4	0·75	12·4	17·6	9·2	15·1
II	56	3·04	26·6	18·2	9·9	4	1·28	18·7	12·6	7·2	16·4
III	26	1·08	14·7	17·7	9·8	7	0·28	6·1	4·2	2·8	12·0
IV	54	3·33	25·6	21·3	12·0	9	0·56	9·8	4·1	2·8	67·2

observations were made 58 days after the beginning of the experiment (Table IIIc). The glycogen storage test was conducted on 13 rats of each group, The total glycogen deposition in the animals receiving no choline was 53 mg., while in those which received choline it was 87 mg. The next observations were made in 70 days after the start of the experiment, and the results are given in detail in Table IV. The average total glycogen deposition in the control animals was 69 mg., while in those which received choline the deposition was 107 mg.

The absorption coefficient of the glucose was determined in this experiment. There was a slight leakage of sugar in two cases in the group of animals which received no choline, and in one of the choline-fed animals. The absorption coefficient in the animals which received no choline was 0·22, and in those

which received choline 0·19. There was considerable variation in the individual figures.

The next observations were made 75 days (Table V) after the beginning of the experiment. The bromsulphalein test was conducted on 18 animals to which no choline had been administered, and on 17 of those which received choline. The dose of bromsulphalein was slightly increased in this experiment, as described under " Methods ". It was found to be positive in 8 cases and negative in 9 of the group which received no choline. In one case the injection was not satisfactory, and this test was not completed. The test was negative throughout on the 17 animals which received choline.

Ketone body production.—Since there is considerable evidence that the liver is the principal site of the production of ketone bodies, it occurred to us that a study of the excretion of these substances during fasting in animals with different amounts of fat in their livers might be interesting. Our attention was focused on this possibility by the results of the experiments of Best and Campbell (1936), in which an increase in liver-fat accompanied the ketosis produced by the injection of certain extracts of the anterior pituitary gland. Four groups of 20 rats with an average weight of approximately 200 g. were placed on the low choline diet for 21, 56, 26 and 54 days respectively. At the end of these periods 10 animals of each group were sacrificed to determine the initial values of liver and body-fat. The remaining 10 of each group were fasted and the average daily ketone excretion for each group was determined. The results for the first three groups are shown in Table VI, and Fig. 1 illustrates the daily excretion of rats in Group IV. Normal male rats excrete only a very small amount of ketones during a 4-days fast. In our experience the average for a 200-g. rat for a 4-day period is about 3 mg.*

The results shown in Table VII (Groups I–III are the same as those referred to in Table VI) indicate that the ketosis was much greater in animals with fatty livers than in normal rats. This phenomenon was first reported by Deuel and his collaborators (1937), and we have had the pleasure of discussing our findings with them. In Group I the initial liver-fat of 10 rats at the beginning of the fast was 14·9 p.c., and in a similar group that had been fasted for 4 days the liver-fat was 12·4 p.c., *i. e.* there was still a large amount of fat in the livers. The total ketone excretion during this period was 15·1 mg. The excretion of ketones began to decrease after the 3rd day of fasting (Table VI). As a rule a transient increase in ketone excretion occurs during the 2nd or 3rd day of the fast. In Group III the observations were extended to 7 days, but there was no significant difference in the excretion after the 3rd day. It would appear that while the excretion of ketones is increased when fat is present in abnormally large amounts in the liver, the extent and duration of the excretion are not proportional to the amount of fat found in that tissue. Two days after the maximum fasting excretion of ketone bodies had been observed in Group II the liver-fat was definitely higher than that of the animals in Group III at the beginning of the fast.

It is apparent from the results discussed above that the explanation of the falling off in ketone excretion is not attributable to lack of substrate in

* We are greatly indebted to our colleague, Mr. J. Campbell, for his co-operation in these experiments.

the liver-cells. It might be due to inability of the hepatic cells to form ketone bodies or to inadequate stimulation of this process. That ketone bodies can still be formed after the point of maximum excretion has passed is established by the results of the following experiment: Rats which had been kept on the diet low in choline for 54 days were fasted (Group IV). The average liver-fat of a control series of 12 rats at the beginning of the fast was 25·6 p.c. The average daily ketone excretion of the 10 fasted rats is shown in Fig. 1. The amounts excreted on the 4th and 5th day of starvation are definitely less than on the 2nd or 3rd. On the 5th day the injection of anterior pituitary extract (Best

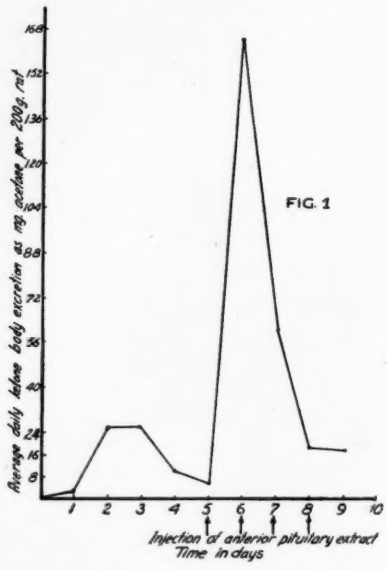

FIG. 1

and Campbell, 1936) was commenced. This was continued in the same dosage for 4 days. The pronounced increase in the excretion of ketone bodies indicates that these substances can still be formed. While the most probable explanation of these findings is that the pituitary extract has stimulated the liver-cells to resume ketone body production at an increased rate, other factors may, of course, play a role. It is interesting that the excretion of ketone bodies diminished in spite of the continued injection of the extract. While the results of this preliminary study on ketosis have thrown no further light on the effect of diets low in choline on liver function, several interesting points which merit further investigation have been uncovered.

DISCUSSION.

In previous papers in which the results of feeding diets deficient in choline to rats were recorded, the irregularity of the deposition of fat in the liver in

short experiments has been frequently discussed. The findings presented in this paper show that much of this irregularity *may* disappear when the observations are made over prolonged periods. This is particularly well demonstrated in Table II. Another point of considerable significance is that the addition of choline to the basal diet caused a much more satisfactory gain in weight than in previous experiments. This result is undoubtedly due to the use of a better protein preparation. It is not suggested, however, that the favourable effects are due only to the protein *per se*. The meat powder exerts less lipotropic effect than carefully prepared casein, and it produces a greater gain in weight. In Table II, for example, the average gain in weight was 17 g., while the average loss in the animals which received no choline was 14 g. The average body-fat of the animals which received choline was 22·5 g., while in those which received none the value was 15·7 g. (The average body-weight of the two series was the same at the beginning of the experiment.) The average total fat, *i. e.* of the body and liver, was 22·8 g. in the choline series and 19·1 g. in the controls. It would appear, therefore, from these results that the addition of choline to a satisfactory basal diet produces an actual increase in the deposition of fat in the bodies of the choline-fed animals. The liver remains free of excessive amounts of fat, while storage in the depots is encouraged. *It may, therefore, be suggested that the presence of choline in the diet favours the normal distribution of fat between the liver and the depots.*

It is remarkable that livers of which the cells appear when examined microscopically to be profoundly degenerated, are capable of storing appreciable amounts of glycogen when glucose is administered, in spite of the presence of very large amounts of fat. There would appear, however, to be definitely less glycogen stored in very fatty livers than in the livers of the choline-fed animals when a certain quantity of sugar is absorbed. It will be appreciated that the concentration of glycogen is always much less in the large fatty livers than in the much smaller ones of the choline-fed animals. It is therefore unsatisfactory to compare, as some workers have done, the concentration of glycogen in the livers under similar conditions. There have been several previous reports on the fact that fatty livers are able to store appreciable amounts of glycogen. We are not familiar, however, with previous attempts to determine the amount of glycogen stored in the fatty liver when a definite amount of glucose is absorbed. The fact that more glycogen is stored in the livers of the choline-fed animals indicates that the provision of choline in the diet prevents a decrease in the glycogenic ability of the liver.

The situation with regard to the retention of bromsulphalein is somewhat similar to that observed in the glycogen storage experiments. No retention of the dye was noted in the animals which received choline, even when they were maintained upon the basal diet for as long as 75 days. The animals receiving the basal diet without choline, however, showed, in an appreciable number of cases, definite retention of the dye. In one experiment, for example, 8 of the 17 animals on the basal diet were unable to excrete the dye under the conditions of the test. These results provide another example of the failure of a function of the liver when animals are maintained on a diet deficient in choline.

An attempt was made to investigate another liver function by estimating

the excretion of ketone bodies in the animals with very fatty livers. We noticed, in confirmation of the findings of Deuel and his collaborators (1937), that the animal with a fatty liver excreted much more ketones than the normal rat. The extent of the excretion, however, was not proportional to the amount of excess fat present in the liver at the beginning of the period of fasting.

We became very much interested in the transient nature of the ketosis (Tables VI and VII). The decrease in excretion of ketone bodies might have been due to failure of the supply of fat to the liver-cells. This was certainly not the case, because an abundance of fat was found there even when the ketone body excretion had fallen to a low level. The decrease might have been due to inability of the liver-cells to form ketones. The results of the experiment illustrated in Fig. 1 show quite definitely that the formation of ketones can be stimulated after the falling off in excretion has been observed. These experiments are of a preliminary nature, however, and since a large dose of pituitary extract was used, we cannot suggest that the livers were not being stimulated by the ketogenic substance of the animal's own pituitary to a normal extent. These latter results really throw no further light on the failure of liver function which sometimes occurs when large amounts of fat are present, but they have provided an interesting opportunity for new studies on ketosis, which are being pursued in collaboration with Mr. J. Campbell.

SUMMARY AND CONCLUSIONS.

1. The extent of deposition of fat in livers of rats on a diet low in choline, which is usually irregular when observations are made for short periods, may become much more uniform when the experimental period is prolonged.

2. The animals which received choline gained in weight and stored fat in their depots while the controls, receiving the same diet without choline, lost weight and retained appreciably smaller amounts of fat in the fat depots.

3. Tests to detect loss of liver function, based on the ability of this organ to store glycogen or to excrete the dye bromsulphalein, have been found to be positive in a certain number of animals with very fatty livers. The tests were uniformly negative in animals which received adequate amounts of choline.

4. The results of preliminary studies in rats with fatty livers indicate that these animals excrete during starvation appreciably larger amounts of ketone bodies than normal rats (confirming Deuel and collaborators), but that the extent of the ketonuria is not proportional to the amount of excess fat present in the liver.

5. Large amounts of fat may be present in the liver after the ketonuria has largely subsided and a further excretion of ketone bodies may then be elicited by the injection of certain extracts of the anterior pituitary gland.

6. The results indicate that the presence of choline in the diet (1) favours the normal distribution of fat between the liver and the body depots, and (2) prevents the failure of certain functions of the liver.

REFERENCES.

Best, C. H., and Campbell, J.—(1936) *J. Physiol.*, **86**, 190.
Cori, C. F., and Cori, G. T.—(1926) *J. biol. Chem.*, **70**, 557.
DeLong, W. A.—(1927) *Ibid.*, **72**, 731.
Deuel, H. J., Jun., Hallman, L. F., and Murray, S. W.—(1937) *Ibid.*, **119**, xxii.

OBSERVATIONS ON THE PLASMA LIPOIDS IN VARIOUS TYPES OF LIPÆMIA (WITH SPECIAL REFERENCE TO RENAL DISEASE).

FREDA K. HERBERT.

From the Pathological Department of the University of Durham College of Medicine and the Royal Victoria Infirmary, Newcastle upon Tyne.

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THE lipoids circulating in the blood may be derived from various sources. If one accepts the view that the liver desaturates fatty acids in preparation for their utilization by other tissues, one would expect that lipoids in process of transport from the liver to the tissues would consist of compounds containing the more unsaturated fatty acids. Fat in process of mobilization from the reserve depots—the adipose tissue, and the "*élément variable*" of other tissues—would yield predominantly the less unsaturated fatty acids. Moreover, lipoids derived from the liver would include phospholipins and cholesterol esters, whereas the adipose tissue would supply glycerides.

In pathological lipæmia the accumulation of lipoids in the blood might occur, predominantly, either at the stage of transport from the liver to the tissues, or at the stage of mobilization from the reserves. A study of the proportions of the various lipoids in the plasma, and of the mean iodine value of the fatty acids, might indicate the stage at which the accumulation occurred. The present work was undertaken with this object in view. Observations have been made in eight normal subjects, in seven cases of renal œdema, in one case of lipæmia in the terminal stage of chronic nephritis, in one case of non-diabetic xanthoma, and in one case of gross diabetic lipæmia with xanthoma. Analyses have been made on plasma only, because in persistent lipæmia the abnormalities are confined to the plasma.

METHODS.

Oxalated blood was collected in the "fasting" state (13–17 hours after food). Large amounts, generally yielding from 15 to 30 c.c. plasma, were used.

The following estimations were made: Total extract, lipoid phosphorus, free cholesterol, total unsaponifiable matter, total cholesterol, total fatty acids, and the iodine value of the fatty acids.

Extraction of Lipoids.

Two methods of extraction have been used. The first has the advantage of giving an extract free from extraneous material; the second, Bloor's, gives a more complete extraction of phospholipin.

First method (described in the tables as Method A).—20 c.c. plasma, or a smaller measured volume diluted to 20 c.c. with water, was placed in a Roehrig tube, the volume below the tap

level being 40 c.c. Absolute alcohol, 20 c.c., was added, bringing the level of the mixture just below the tap. Ether, 20 c.c., was added, and the mixture thoroughly shaken. Light petroleum, 25 c.c., was added and the mixture again shaken. The layers were allowed to separate, and the supernatant extract was run off through a fat-free filter into a weighed light flask. A second extraction was made with 20 c.c. ether and 20 c.c. light petroleum, and a third extraction with 15 c.c. of each solvent, the extracts being collected in the same flask. The tap and filter were washed with light petroleum and the washings combined with the extract. The solvent was removed by distillation, followed by storage of the residue in a vacuum desiccator until constant weight was reached. From this the weight of " total lipoids " was calculated. The extract was then dissolved in hot absolute alcohol, and transferred quantitatively to a volumetric flask (50 or 100 c.c. according to the amount of the extract), cooled, and made to volume with alcohol. An aliquot part of this solution was taken for the estimation of lipoid phosphorus, a second aliquot part for the estimation of free cholesterol, and a third for saponification, and the separation and estimation of unsaponifiable matter, total cholesterol, and fatty acids, as described below.

Second method (described in the tables as Method B).—The plasma (15 to 30 c.c.) was extracted by Bloor's method. Suitable aliquot parts of the filtrate were set aside for the determination of lipoid phosphorus, and of free cholesterol. Another aliquot part, usually 250 to 275 c.c., was used for the remainder of the analyses. The solvent was removed from this sample by distillation under reduced pressure in an atmosphere of nitrogen. The residue was extracted with successive portions of ether, the ether solution filtered, and the combined ether solution and washings made to 100 c.c. Aliquot parts of this solution were set aside for the determination of the phosphorus soluble in ether at this stage. The remainder of the solution was transferred quantitatively to a weighed flask. The ether was removed by distillation, and the residue stored in a vacuum desiccator until constant weight was reached. From this weight the total extract was calculated. The sample of lipoids was then used for the separation of unsaponifiable matter and total fatty acids, and for analysis of these fractions.

Analytical Methods.

Saponification and separation of unsaponifiable matter and fatty acids.—The sample of extract in alcoholic solution (usually containing 0·07 to 0·20 g. extract) was saponified by heating with sodium ethylate under a reflux condenser for 2–3 hours. The resulting mixture was transferred to a separating funnel, first alcohol and then water being used, so that after complete transference the mixture contained approximately equal parts of water and alcohol. The unsaponifiable matter was removed by three successive extractions with a mixture of equal parts of ether and light petroleum. The combined solution containing the unsaponifiable matter was washed at least three times by shaking with water, the earlier washings being added to the main solution of soaps. The solvent was removed from the unsaponifiable matter and the extract weighed. The alcohol-water solution containing the soaps was acidified, and the fatty acids extracted with ether and petroleum, by three successive extractions. The combined solution was washed with water, the solvent removed and the extract weighed.

The extract of unsaponifiable matter was used for the estimation of total cholesterol, and the extract of fatty acids for the determination of iodine value.

Cholesterol was estimated gravimetrically as digitonide by the method of Gardner and Gainsborough (1927). Free cholesterol was estimated on a sample of the unsaponified blood lipoids in alcohol solution. Total cholesterol was estimated in an alcoholic solution of the unsaponifiable matter. The difference between the total cholesterol and free cholesterol gives the cholesterol combined as ester. For the calculation of the cholesterol ester (expressed as ester) it is assumed that the fatty acids combined with cholesterol have a mean molecular weight of 291. This figure for the molecular weight of fatty acids of plasma is derived from the work of Wilson and Hansen (1936).

Lipoid phosphorus.—The solution of blood-lipoids was evaporated to dryness and the residue digested with perhydrol and sulphuric acid. After the removal of the peroxide the phosphorus in the digest was estimated either by the method of Kuttner and Lichtenstein (1930) or that of Stewart and Hendry (1935). The methods agreed well, but the latter is more convenient for this type of work. Phospholipin was calculated on the assumption that the phosphorus amounts to 4 p.c. of the phospholipin.

Iodine value.—The iodine value of the separated fatty acids was estimated by Wijs' method.

Notes on the Two Extraction Methods.

In the first extraction method, the alcohol is used to dissociate lipoid-protein complexes, but is not used as an extracting solvent; the extract should, therefore, be free from alcohol-soluble substances other than the lipoids. In the Bloor method, on the other hand, it is well known that the crude extract contains much non-lipoid material, including salts and nitrogenous substances. To obtain a direct determination of total lipoids in a Bloor extract it is necessary to separate the lipoids from the extraneous material; this was attempted by removing the alcohol and ether as already described, and estimating the ether-soluble material in the residue.

The purity of the extracts obtained may be judged by the proportion of the total extract recovered after saponification. Saponification, and subsequent removal of water-soluble substances, will cause loss of glycerol, choline and phosphoric acid. On the assumption that the phospholipin is lecithin, the expected loss can be calculated for any sample from the phospholipin figure. (The loss of glycerol from glyceride is neglible in relation to total lipoids.)

For the extracts obtained by " method A " the results were as follows: The sum of the unsaponifiable matter and the fatty acids ranged from 89 to 96·9 p.c. of the total extract. The amount lost during saponification and washing was from 3·1 to 11·0 p.c. of the total extract, with a mean value of 7·2 p.c. The calculated loss for the same series of samples ranged from 4·8 to 10·9 p.c., with an average of 8·6 p.c. On average figures, there is no significant discrepancy between the actual and the expected loss. This proves the absence of any appreciable amount of extraneous material, and also suggests that all the phospholipin in these extracts is lecithin or kephalin, since if sphingomyelin were present the loss would be greater.

For the ether extract derived from a Bloor extract, the sum of the unsaponifiable matter and fatty acids ranged from 68 to 88 p.c. of the total extract (average 78 p.c.). It is possible that the Bloor extract may include sphingomyelin, but even so, the loss during saponification and washing is too great to be accounted for by the removal of the water-soluble products of saponification of the phospholipins. Even in the ether extract there must be some non-lipoid material, and the method is less selective than that first described.

As regards the completeness of the extraction, the Bloor method has apparently some advantage. The two extraction methods agree well for cholesterol and fatty acids, and for the iodine value of the fatty acids. The Bloor method gives a rather higher figure for lipoid phosphorus. If one takes the total phosphorus of the original Bloor extract as the correct figure, the other method extracts from 76 to 103 p.c., with an average of 86 p.c.

But this raises the question whether the total phosphorus of a Bloor extract is all lipoid phosphorus. In the course of the present work with Bloor extracts from plasma, it was found that after transference of the lipoids to ether solution, the phosphorus of the original extract was never completely recovered. The loss was diminished by distilling off the solvents under reduced pressure, but under the conditions of the writer's experiments it was not prevented altogether. The phosphorus recovered ranged from 61 to 92 p.c. of the original phosphorus, with an average of 80 p.c. In most of these experiments the temperature was allowed to rise to about 90° C. for a short time during the later part of the distillation; but it was found that the loss was not prevented when the temperature was kept below 60° C. throughout. The phosphorus which failed to dissolve in ether was mainly in the form of water-soluble organic phosphorus; there was only a trace of inorganic phosphate. The ether extract contained traces of free fatty acid. This suggested that the main cause of loss might be hydrolysis of part of the phospholipin in the original extract during distillation.

Page, Kirk, Lewis, Thompson and Van Slyke (1935) removed the alcohol and ether from Bloor extracts at a temperature below 60° C. and re-dissolved the lipoids in petroleum. They found that 20 to 30 p.c. of the original phosphorus was lost in this manipulation, but interpreted this as showing the presence of non-lipoid phosphorus in the original extract, and based their calculations of phospholipin on the petroleum-soluble phosphorus. Ellis and Maynard (1937), working with bovine plasma, found gross losses of phosphorus in transferring the lipoids from a Bloor extract to ether or petroleum solution, but were able to prevent the loss entirely by removing the alcohol and ether from the Bloor extract at a temperature below 40° C. This confirms the view that the whole of the phosphorus of a Bloor extract is lipoid phosphorus, and that decomposition of phospholipin occurs during distillation carried out above 40° C.

Stewart and Hendry (1935) showed that, with whole blood, the total phosphorus of a Bloor extract could be recovered in ether solution and could then be precipitated with acetone and

magnesium chloride. But in a personal communication, Stewart says that he has also had difficulty in manipulating plasma lipoids, and found losses similar to those already described. It would appear that decomposition of the plasma phospholipin may be prevented in the presence of corpuscle constituents. Thannhauser and Setz (1936) found that the phospholipins of serum included a considerable proportion of sphingomyelin. The sphingomyelin was present in a Bloor extract, but a large part of it was lost during removal of the alcohol and ether from the extract; this was attributed to volatilization of the phospholipin.

Upon the whole, the evidence shows that the phosphorus of a Bloor extract is all lipoid phosphorus, and in the present work the phospholipin figures for samples extracted by "method B" have been calculated from this total phosphorus. If the loss of phosphorus during distillation is due to hydrolysis of phospholipin, no error will be introduced into the estimation of total fatty acids. If it is due to loss of part of the sphingomyelin the error due to loss of sphingomyelin fatty acids will be very small in relation to the total fatty acids.

Total lipoids and glyceride.—As has been shown, extracts obtained by "method A" are free from any appreciable amount of extraneous material, and the direct estimation of the total extract gives a valid figure for the total lipoids.

Where "method B" has been used, the ether extract contains extraneous material. The figure for "total extract" has therefore been disregarded and the total lipoids have been calculated from the sum of the fatty acids and unsaponifiable matter; a correction has been made for the water-soluble products of saponification of the phospholipin. This method unfortunately involves an assumption as to the nature of the phospholipin mixture. For lecithin, the water-soluble fraction amounts to 29·4 p.c. of the phospholipin. In view of the uncertainty as to the proportions of mono- and diaminophosphatide in any given sample, especially in pathological cases, it was decided to use this arbitrary factor, bearing in mind that if sphingomyelin is present the results for total lipoid and glyceride will be too low.

Glyceride was calculated by difference. The sum of the phospholipin, free cholesterol, cholesterol ester and the non-sterol unsaponifiable matter was calculated, and subtracted from the total lipoid.

THE NORMAL PLASMA LIPOIDS.

The data for normal subjects are given in Table I.

Although large numbers of analyses of the normal plasma lipoids are available in the literature, these results have some special interest on account of the methods employed. Sufficient material was used to enable gravimetric methods to be employed for the estimation of cholesterol, and to allow of the purification and gravimetric estimation of fatty acids and unsaponifiable matter. The use of the separated fatty acids has obvious advantages for the estimation of iodine values. The method of extraction described as "method A" gives a purer extract than the more usual methods of extraction, and although more work is required in regard to the phospholipins extracted, it seems that the principle of using alcohol to dissociate lipoid-protein complexes, but not as an extracting solvent, has possibilities which may usefully be further explored. The glyceride figures given by this method are probably more reliable than those obtained in any other way, as no arbitrary assumptions are involved.

The figures obtained for total fatty acids, phospholipin and total cholesterol are in general agreement with the results of other authors working with modern methods. The series includes one case (Case 1) in which the total cholesterol is rather high according to the standards which have been generally accepted in the past, but it comes within the normal range recently reported by Sperry (1936).

The figures for free cholesterol, and the ratio of free to total cholesterol, require comment. The range for free cholesterol in the present series is from

TABLE I.—*Plasma Lipoids in Normal Subjects.*

(Percentage of Plasma.)

Case.	Extraction method.	Total lipoids.	Total fatty acids.	Phospholipin.	Free cholesterol.	Total cholesterol.	Cholesterol ester (as ester).	Non-sterol unsaponifiable matter.	Glyceride.	Iodine value of fatty acids.
1	A	0·758	0·389	0·260	0·113	0·302	0·327	0·036	0·022	91
2	A	0·433	0·246	0·154	0·053	0·159	0·183	0	0·043	93
3	A	0·591	0·322	0·209	0·068	0·201	0·230	0·029	0·055	79
4	B	0·566	0·296	0·201	0·057	0·182	0·216	0·029	0·063	117
5	B	0·509	0·260	0·183	0·053	0·130	0·133	0·065	0·075	110
6	B	0·518	0·267	0·177	0·070	0·158	0·152	0·041	0·078	120
7	B	0·540	0·267	0·199	0·052	0·147	0·164	0·067	0·058	109
8	B	0·612	0·320	0·209	0·041	0·206	0·285	0·025	0·052	124
Average		0·566	0·296	0·199	0·063	0·183	0·211	0·037	0·056	105

0·041 to 0·113 p.c. of the plasma, with an average of 0·063, and the free cholesterol constitutes from 20 to 44 p.c. of the total cholesterol, with an average of 34 p.c. The results are in general agreement with those reported by Gardner and Gainsborough (1927), and were obtained by the same method. Sperry (1936) has found a much more constant ratio of free to total cholesterol. He used the method of Schoenheimer and Sperry, which is based on the application of the colorimetric method to the digitonide precipitate. In 126 samples he found that the free cholesterol constituted from 24 to 30 p.c. of the total cholesterol, with an average of 26·9 p.c. Page, Kirk, Lewis, Thompson and Van Slyke (1935) obtained higher values for free cholesterol than the other authors mentioned.

In the present work a small amount of material other than cholesterol has usually been found in the unsaponifiable matter. Many authors have found such a fraction. Gardner and Gainsborough (1934) regard it as an artefact derived from various sources. They suggest, firstly, grease from the taps of separating funnels; secondly, ether-soluble material derived from sodium ethylate; and thirdly, ether-soluble material derived from partial hydrolysis of soaps during the extraction of the unsaponifiable matter. The values found for the non-sterol fraction in the present work are lower than those for the material used by Gardner and Gainsborough. In this work no tap-grease has been used, and care has been taken to exclude extraneous material. The extraction of unsaponifiable matter was made from 50 p.c. alcohol solution, and this should prevent hydrolysis of soaps at this stage. Pigment passes into the unsaponifiable fraction, and this is probably derived from the plasma (carotene and xanthophyll). The non-sterol fraction, in chloroform solution, gives only a brownish colour with acetic anhydride and sulphuric acid. The possibility of a small amount of extraneous material cannot be entirely excluded, but it is probable that there is a genuine non-sterol fraction.

In the present series of analyses the figures for glyceride are lower than those found by other authors. Glyceride can only be estimated as the undetermined residue after other constituents of the total lipoid have been allowed for, and the results are therefore liable to error arising from the summation of the technical errors of all other determinations. With the usual methods of estimation of total lipoids, other sources of error also arise. Page *et al.* (1935) reported figures for the "neutral fat" of normal plasma ranging from 0·088 to 0·362 p.c., with an average of 0·225 p.c., though they make it clear that the so-called neutral fat includes other substances than glyceride. They took the total petroleum-soluble material derived from a Bloor extract as a measure of total lipoids. They pointed out that this material included unknown nitrogenous substances. It would also include the non-sterol unsaponifiable matter, which would be included with the "neutral fat". Boyd (1933) estimated the glyceride fatty acids as the residual fatty acids left after allowing for the fatty acids of phospholipin and cholesterol ester, and obtained figures for glyceride ranging from 0·078 to 0·229 p.c. of the plasma, with an average of 0·154 p.c. His method of calculation involved the assumption that the phospholipin fatty acids constituted 67 p.c. of the phospholipin, and this was supported by some direct determinations of phospholipin fatty acids. But the phospholipins included were the ether-soluble phospholipins derived from a

Bloor extract. If some phospholipin was lost in transference from the original extract to ether solution, the fatty acids corresponding to the lost phospholipin would be calculated as glyceride fatty acid.

As regards the present work, the glyceride figures obtained by " method B " depend on the assumption that all the phosphorus of the original Bloor extract is phospholipin phosphorus, and that the phospholipin is a mixture of lecithin and kephalin. The range for this method is from 0·052 to 0·078 p.c. of the plasma. If sphingomyelin is present, these figures will be too low. If they are recalculated on the basis of the average proportions of mono- and diamino-phosphatide found by Thannhauser and Setz, the glyceride figures range from 0·087 to 0·106 p.c. But the low level of plasma glyceride is confirmed by the figures obtained by " method A ". Here no assumption is made as to the nature of the phospholipin mixture ; the total lipoids are determined directly in an extract which is purer than those obtained by other methods. Since only three estimations have been made on normal plasma by this method, the normal range cannot be fixed, but all three estimations showed low glyceride figures (0·022, 0·043, 0·055 p.c.).

From the present results it has been calculated that normally the fatty acids of phospholipin account for from 44 to 52 p.c. of the total fatty acids, the fatty acids of cholesterol ester from 22 to 39 p.c., and the fatty acids of glyceride from 5 to 28 p.c.

The iodine values of the separated fatty acids range from 79 to 124, with an average of 105. This is in general agreement with other data in the literature. Figures obtained on separated fatty acids by other authors are : 99 to 113 (Wilson and Hansen, 1936), 100 to 136 (Hansen, 1933), and 106 to 154* (Ling and Liu, 1928). Other data have been obtained indirectly, by determining the iodine value of an extract containing cholesterol and fatty acids, and making the necessary correction for the iodine uptake of cholesterol. This calculation is only valid when the Rosenmund-Kuhnhenn method is used for the estimation of iodine value. By the indirect method Boyd (1933) found the mean iodine value of the fatty acids of normal plasma to range from 65 to 102, with an average of 88·5, and Stoesser (1936) found a range of 83 to 143.

THE PLASMA LIPOIDS IN RENAL DISEASE.

Clinical Material.

The series includes examples of glomerulo-tubular nephritis in all stages, and also two cases which may be pure lipoid nephrosis, so far as one can judge from clinical evidence alone. The presence of lipoid deposits in the kidneys was confirmed in most of the cases by the examination of the urine deposit under the polarizing microscope. Sphero-crystals, in epithelial cells, and lying free, were observed in Cases 10, 11, 12, 13, 15, 16 and 17. In the other three cases this examination was omitted.

Full clinical data will not be given, but the following notes will serve to indicate the classification of the cases and the stage of the disease.

* Figures recalculated to give the mean iodine value of total fatty acids.

Case 9.—Male, aged 15. Acute hæmorrhagic nephritis, with slight œdema early, becoming severe and generalized later. Blood-pressure 176/80. Blood-urea on admission 108 mg. per 100 c.c., rising to 164 a month later. Serum protein on admission 5·12 p.c. The analysis of blood lipoids was made 7 weeks after the onset of symptoms.

Case 10.—Male, aged 16. The patient had mitral valvular disease of the heart as a result of rheumatic infection, but the cardiac lesion appeared to be compensated. He developed nephritis, with slight hæmaturia, and gross œdema and ascites. Blood-pressure 130/78. Blood-urea 30 to 36 mg. per 100 c.c. Blood-urea clearance 29 p.c. of normal average. Death occurred 4 months after the onset of renal œdema. The post-mortem examination confirmed the clinical diagnosis, and the kidneys showed intra-capillary glomerulitis and extensive lipoid deposits in the tubular epithelium. The results of analysis of the kidneys are given later in this paper.

Case 11.—Male, aged 21. Recurrent attacks of hæmorrhagic nephritis during three years. The patient was admitted in an attack, in which he had œdema for the first time, slight in degree. Blood-pressure 190/120. Blood-urea clearance 7·5 p.c. of normal average. Urea concentration test: maximum urine urea 1·72 p.c.

Case 12.—Male, aged 22. The patient was first seen in an attack of acute hæmorrhagic nephritis with uræmia. His condition improved temporarily, but the disease passed into the chronic stage, and he died of uræmia 5 months after the first attack. There was transient œdema in the acute stage only. The analysis of plasma lipoids was made six weeks after the onset of the disease, when the œdema had already subsided.

Case 13.—Female, aged 24. The disease began with an attack of acute hæmorrhagic nephritis, which passed into the chronic stage with recurrent œdema. The case was studied a year after the first attack, during an exacerbation associated with hæmaturia and generalized œdema. Blood-pressure 145/105. Blood-urea 26 mg. per 100 c.c. Serum protein 3·41 p.c.

Case 14.—Male, aged 68. The patient came under observation 4 months after the onset of symptoms. There was no history of hæmaturia, and only a few red cells were seen on microscopic examiantion of the urine deposit. There was gross œdema of the legs and trunk and free fluid in the peritoneal and pleural cavities. Blood-pressure 145/75. The blood-urea on admission was 36 mg. per 100 c.c. It steadily rose, and reached 160 mg. per 100 c.c. a month after the patient's admission. Serum protein 4·5 p.c.

Case 15.—Male, aged 18. The patient had had rheumatic fever. The renal disease began insidiously with slight œdema at first, increasing later. The patient was observed during two periods in hospital. In the first period œdema was absent so long as he remained in bed. There was no hæmaturia. There was albuminuria, and the urine deposit showed renal epithelial cells and hyaline and granular casts. The blood-urea clearance was 100 p.c. of normal. The serum protein was 6·88 p.c. The first analysis of blood lipoids was made at this stage. On the second admission 6 months later there was generalized œdema and ascites. Blood-pressure 130/80. Blood-urea clearance 63 p.c. of normal. The second analysis of blood lipoids was made during this period.

Case 16.—Female, aged 31. The patient had been under treatment for syphilis for 6 months. The onset of renal disease was marked by the development of generalized œdema and ascites, with no hæmaturia. Blood-pressure, systolic, 120. The blood-urea was raised early (60 to 76 mg. per 100 c.c.) and fell later. The blood-urea clearance, three weeks after the onset of symptoms was 54 p.c. of the normal average; a month later it had risen to 89 p.c. The serum protein ranged from 3·8 to 3·6 p.c. The lipoid analysis was made a month after the onset of symptoms.

Case 17.—Female, aged 19. The patient was observed during two periods in hospital. Albuminuria had first been noted 7 months before the first admission, and during that period the patient had been on a low-protein diet. She had generalized œdema and ascites, which improved considerably during her first stay of 7 weeks in hospital, but soon recurred and again became severe. She returned to hospital 6 weeks after discharge. There was never any gross hæmaturia, and only a few red cells in the urine deposit. The blood-urea ranged from 17 to 35 mg. per 100 c.c. and the blood-urea clearance was normal. Blood-pressure 115/70. Serum protein 4·16 to 3·20 p.c. The first analysis of blood lipoids was made a month after the first admission, the second 6 weeks later.

Case 18.—Male, aged 30. The symptoms began insidiously with malaise and attacks of tetany. There was no history of hæmorrhagic nephritis or œdema. The urine showed albuminuria and microscopically a few red cells and casts. Blood-urea 256 mg. per 100 c.c. Serum calcium 4·9 mg. per 100 c.c.

Lipoid Analyses in Renal Disease.

The results are given in Table II.

The best examples of lipæmia associated with the " nephrotic syndrome " are Cases 13 to 17. In these, all the plasma lipoids are increased—the glyceride and phospholipin as well as the cholesterol and cholesterol ester. These results agree with those of Page, Kirk and Van Slyke (1936).

In general the glyceride shows proportionally the greatest increase, the cholesterol and cholesterol ester come next in order, and the phospholipin is relatively less increased. In Cases 13 to 17 the fatty acids of phospholipin constitute from 16 to 44 p.c. of the total fatty acids ; the fatty acids of cholesterol ester, 19 to 36 p.c., and the fatty acids of glyceride 21 to 54 p.c.

The free cholesterol forms an approximately normal proportion of the total cholesterol. The range is from 19 to 36 p.c. in Cases 13 to 17. This is in agreement with the results of Gainsborough (1929). Lichtenstein and Epstein (1931) reported an increased proportion of cholesterol ester in such cases ; this has not been found in the present series.

The iodine values of the separated fatty acids mostly fall within normal limits ; there is a tendency to figures below the normal average, but with so few cases one cannot attach any significance to this. Ling and Liu (1928) reported low iodine values in " nephrosis ". If their figures are recalculated to give the mean iodine values of the total fatty acids, their results are, for normal subjects, 106 to 154, and in " nephrosis " 54 to 73. No such sharp distinction has been found in the present work. The degree of lipæmia in the Chinese series was no greater than in the writer's cases.

Case 18 of the present paper shows lipæmia with a predominance of fatty acids of low iodine value. The cholesterol figures are normal, and the ratio of total fatty acids to total lipoids indicates a high proportion of glyceride. The patient was in the terminal stage of glomerulo-tubular nephritis, with uræmia, and had never had œdema.

Kidney Analysis in Case 10.

In Case 10 the plasma lipoids showed no significant increase, but there were extensive lipoid deposits in the kidney. Microscopically the kidney showed intracapillary glomerulo-tubular nephritis, with large amounts of lipoid in the renal epithelium.

The right kidney, which weighed 6¾ oz., was used for chemical analysis. The extensive lipoid deposits in the cortex made it easy to distinguish cortical from pyramidal tissue, and only the cortex was analysed. The tissue was cut small, ground with sand and mixed with an excess of anhydrous calcium sulphate. The drying was completed in a vacuum desiccator. Successive portions of the mass were extracted with ether in a Soxhlet apparatus for 4 hours. The ether was removed and the extract analysed by the methods already described for plasma extracts. This method of extraction would not remove all the lipoids from normal tissues, but in the material here used there was a pathological accumulation of lipoid in the form of free droplets, and this abnormal lipoid would be well extracted. A separate sample of the moist tissue was dried in an air oven for estimation of the dry weight of the tissue.

The results were as follows : Total lipoids formed 3·647 p.c. of the fresh tissue and 18·32 p.c. of the dried. The total fatty acids amounted to 37·92 p.c.

TABLE II.—*Plasma Lipoids in Renal Disease.*

(Percentage of Plasma.)

Case.	Diagnosis.	Extraction method.	Total lipoids.	Total fatty acids.	Phospho-lipin.	Free cholesterol.	Total cholesterol.	Cholesterol ester as ester.	Non-sterol unsaponi-fiable matter.	Glyceride.	Iodine value of fatty acids.
9	Subacute nephritis	A	0·575	0·293	..	..	0·182	..	0·050	..	94
10	Ditto	A	0·748	0·403	..	0·101	0·236	0·234	0·007	..	95
11	Chronic nephritis	A	0·751	0·410	0·164	0·095	0·267	0·298	0·007	0·168	89
12	Subacute nephritis	B	0·994	0·471	0·308	0·092	0·309	0·378	0·134	0·082	105
13	Chronic nephritis	B	1·197	0·617	0·388	0·147	0·418	0·469	0·048	0·149	109
14	Ditto	A	1·254	0·700	0·357	0·193	0·367	0·301	0·010	0·321	81
15	"Nephrosis"	A	1·204	0·648	0·149	0·117	0·423	0·529	0·058	0·351	104
		A	1·545	0·887	0·418	0·174	0·469	0·510	0·106	0·337	70
16	Subacute nephritis	B	1·664	0·931	0·417	0·156	0·489	0·578	0·121	0·392	95
17	"Nephrosis"	B	1·623	0·961	0·505	0·146	0·449	0·525	0·065	0·382	96
		B	2·225	1·413	0·476	0·185	0·566	0·659	0·106	0·799	85
18	Chronic nephritis, terminal stage	A	1·367	1·033	..	0·087	0·206	0·205	0·054	High	68
		A	0·957	0·622	..	0·071	0·210	0·240	0·052	„	74

TABLE III.—*Plasma Lipoids in a Case of Diabetic Lipæmia with Xanthoma.*

(Percentage of Plasma.)

Days in hospital.	Total lipoids.	Total fatty acids.	Phospholipin.	Free cholesteol.	Total cholersterol.	Cholesterol ester.	Glyceride.	Non-sterol unsaponi-fiable matter.	Iodine value of fatty acids.
1	15·53	12·905	0·835 (approx.)	0·887	1·493	1·048	12·53	0·236	73
3	2·963	2·006	..	0·300	0·800	0·865	..	..	74
21	0·920	0·548	0·226	0·082	0·256	0·301	0·261	0·028	95

of the total lipoids, and the total unsaponifiable matter to 62·05 p.c. The whole material was therefore accounted for; there was no measurable loss during saponification.

The total cholesterol was 58·7 p.c. of the total lipoid, with free cholesterol 1·1 p.c. The combined cholesterol was therefore 57·6 p.c., and since the mean molecular weight of the fatty acids was 245, the cholesterol ester amounted to 91·5 p.c. of the total extract. The fatty acids combined with cholesterol amounted to 36·6 p.c. of the total extract, so that the fatty acid in other forms could not be more than 1·3 p.c. There was a trace of free fatty acid, which probably accounted for this small fraction.

The percentage composition of the total lipoids was as follows: Cholesterol ester 91·5, free cholesterol 1·1, non-sterol unsaponifiable matter 3·4, phospholipin 1·4, free fatty acid 1·3 (probably), total 98·7. The iodine value of the separated fatty acids was 90·4.

A CASE OF XANTHOMA (NON-DIABETIC).

The patient had generalized cutaneous xanthoma and there was no evidence of diabetes or of renal disease.

The results of analysis of the plasma were as follows: Total lipoids 2·561 p.c., total fatty acid 1·522 p.c., phospholipin 0·628 p.c., free cholesterol 0·301 p.c., total cholesterol 0·854 p.c., cholesterol ester (calculated as ester) 0·957 p.c., glyceride 0·675 p.c., iodine value of fatty acids 100. There was therefore a marked lipæmia with increases in all the constituents of the lipoid extract. The glyceride was increased relatively more than the other constituents, and the glyceride fatty acids accounted for 44 p.c. of the total fatty acids. The type of lipæmia is similar to that already described as associated with renal œdema, and the iodine value of the fatty acids is normal.

A CASE OF DIABETIC XANTHOMA.

The patient had been under treatment for diabetes, but on several occasions departed from his prescribed diet, and relapsed into severe diabetic ketosis with gross lipæmia. Studies made during three such breakdowns have been reported in an earlier paper (Herbert, 1935, Case R. W—). Another breakdown occurred after the publication of those results, as a result of the omission of insulin, and on this occasion there was generalized xanthomatosis. The lipæmia and xanthomatosis cleared up under treatment. The findings for the plasma lipoids in the fasting state are given in Table III.

At the height of the lipæmia the total lipoids were 15·53 p.c. of the plasma, and the glyceride amounted to 12·53 p.c. of the plasma, or 80·7 p.c. of the total lipoids. Phospholipin, free cholesterol and cholesterol ester were all very high, though relatively much less increased than the glyceride. The free cholesterol was 59·4 p.c. of the total cholesterol—a high proportion. At the height of the lipæmia, when there was a gross increase in glyceride, the iodine value of the separated fatty acids was 73. Two days later the iodine value was still low, in spite of a great reduction in the lipæmia, but at the time of the third analysis, when the cholesterol and phospholipin were practically

normal and the glyceride had fallen to 0·261 p.c., the iodine value of the fatty acids had risen to 95.

DISCUSSION.

Many problems relating to the normal intermediary metabolism of fat are still controversial, and any discussion based on current theories must be made with the reservation that the theoretical basis is not yet secure. But it may be supposed that the two main sources of lipoids in circulation in the blood are the reserve stores (including the adipose tissue fat, and the "*élément variable*" of other tissues) and the lipoids of the liver.

It is believed that the liver normally desaturates fatty acids, and that the more unsaturated fatty acids are used in the synthesis of phospholipins. Such phospholipins are supposed to play an important part in the supply of fatty acids to other tissues. But the liver contains also cholesterol esters and glycerides, and the glycerides of the liver may include fatty acids of a relatively high iodine value. Lipoids mobilized from the liver might include phospholipin, cholesterol ester and glyceride. The mean iodine value of the fatty acids of the normal liver is 127. If lipoids in circulation were derived entirely from the liver, their fatty acids should have a mean iodine value of this order.

In adipose tissue the fat is almost all glyceride, with an iodine value of from 60 to 70. The "*élément variable*" of the other tissues is believed to contain fatty acids of a similar iodine value. Muscles have a large "*élément variable*", and yield fatty acids with a mean iodine value of 74 (Cathcart and Cuthbertson, 1931). In any condition associated with the mobilization of reserve fat, one would expect to find a high proportion of glyceride and a tendency towards low iodine values; the lower limit of the iodine value would be that characteristic of reserve fat.

The "*élément constant*" of the tissues is regarded as an essential constituent of cell structure, mainly composed of phospholipins with fatty acids of high iodine value. One would not expect these compounds to come into circulation in the course of intermediary fat metabolism. They might reach the circulation in conditions associated with necrosis or atrophy of tissues, but such a possibility does not arise in the conditions studied in the present work. It must be remembered, however, that there may be other tissue phospholipins beside those of the "*élément constant*".

It would seem, then, that the lipoids in circulation in the fasting state might be derived from the liver, or from the reserve stores. If there were a pathological accumulation of lipoids mobilized from the liver, one might expect to find a relatively low proportion of glyceride, and an iodine value approaching 127. If lipæmia were due to mobilization of reserve fat, one might expect a high proportion of glyceride and an iodine value falling towards 65. But such changes would only be clearly demonstrable if there were a very clear-cut distinction between the two types of lipæmia. A moderately increased proportion of reserve fat would have only a slight effect on the mean iodine value of the mixed fatty acids; and in fact the normal range of iodine values is so wide that the most one could expect would be that a gross preponderance of liver lipoid would be associated with iodine values in the upper part of the

normal range, and a gross preponderance of reserve fat would be associated with iodine values in the lower part of the normal range.

A consideration of normal results alone shows that it is difficult to account for the findings by postulating a particular mixture of liver lipoids and reserve fat. Normally it appears that the glyceride fatty acids form from 5 to 28 p.c. of the total fatty acids. If the glyceride fatty acids had an iodine value of 65, and the remaining fatty acids a mean iodine value of 130, the range of the mean iodine value of the total fatty acids should be from 111 to 125. Many of the data are of this order, but lower values occur which are not specially related to high proportions of glyceride. The range of iodine value in the present normal series is from 79 to 124, and the whole range found in the literature is from 65 to 154. Clearly the fatty acids of lower iodine value are not confined to the glyceride fraction. There is little direct evidence in the literature as to the distribution of the saturated and unsaturated fatty acids between the different lipoid fractions. Boyd (1933), working with human plasma, found that the iodine value of the phospholipin fatty acids was higher than that of the fatty acids of the acetone-soluble lipoids. Bloor (1924), working with the plasma of the ox and pig, came to the conclusion that the cholesterol ester fraction contained the greatest proportion of unsaturated acids, and found surprisingly low values for phospholipin fatty acids (39 to 87, with an average of 71).

In considering pathological lipæmias, one finds that many different conditions show a similar type of lipæmia, namely, an increase in all the lipoids of the plasma, the greatest increase being in the glyceride, the cholesterol and cholesterol ester coming next in order, and the phospholipin showing relatively less increase. Bloor (1921), observed these features in diabetic lipæmia and in the lipæmia which follows hæmorrhage in rabbits. The present work shows that the same general relations hold also in the lipæmia of the " nephrotic syndrome " and in non-diabetic xanthoma. When the lipæmia is moderate in degree, the increased proportion of glyceride fatty acids is not sufficient to cause a very great fall in the mean iodine value of the total fatty acids, even if one assumes that all the glyceride fatty acids have an iodine value of about 65. It is not surprising, therefore, that in the moderate degree of lipæmia which occurs in the " nephrotic syndrome " and in the writer's case of non-diabetic xanthoma, the mean iodine values of the total fatty acids are not significantly lowered.

In gross diabetic lipæmia there may be an overwhelming preponderance of glyceride, and in such cases the iodine value of the fatty acids tends to be low. The following iodine values have been found : 73 (Imrie, 1915) ; 60·6 (Fischer, 1903) ; 68·5, 86·8, 54·5 (Herbert, 1935) ; and 73 (the present work). The total lipoids in the cases mentioned ranged from 9 to 22 p.c. of the plasma (except in Fischer's case, where the figure was 23 p.c. of the whole blood). These results indicate the presence of a high proportion of glyceride derived from the reserves, but the difference between these cases and the moderate lipæmias is probably one of degree. One may say that in many types of lipæmia there are increases in all the plasma lipoids, that the glyceride tends to form an increased proportion of the total lipoid, even in lipæmia of moderate degree, and that in gross diabetic lipæmia this preponderance of glyceride becomes so great that

the mean iodine value of the total fatty acid is determined mainly by the glyceride fatty acids.

It is possible that, whatever the initial failure in function which leads to lipæmia, secondary changes may occur which affect other stages in fat metabolism. A primary failure in utilization of lipoids by the tissues might, in the first instance, cause an accumulation of lipoids mobilized from the liver; but an additional effect might be the mobilization of fat from the reserves. Or an increase in transport from the reserves might cause also increased uptake by the liver, and, with a healthy liver, might lead to an increased output of liver lipoids. If such sequences occur, it will be impossible to discover the primary site of the disturbance by a study of the plasma lipoids.

As regards the relation between the lipæmia of the " nephrotic syndrome " and the lipoid deposits in the kidney, the present work affords one example of a comparison between the plasma lipoids and the kidney lipoids (Case 10). Here there was only an insignificant increase in the plasma lipoids, whereas the kidney contained pathological lipoid deposits consisting almost entirely of cholesterol ester. The total lipoids extracted by ether amounted to 18·32 p.c. of dry weight of the kidney, and of this material, 91·5 p.c. was cholesterol ester. This amount of cholesterol is far too large to have been the result of degeneration of the cells with liberation of their lipoid. It must have been due to infiltration of the cells with lipoid taken up either from the blood or from the glomerular filtrate. This apparently occurred without any great increase in blood cholesterol, but as the lipoid analysis was made three months after the onset of renal œdema, it is possible that hypercholesterolæmia occurred at an earlier stage. But if the accumulation of lipoids came from the blood, it is difficult to understand why the cholesterol ester was selectively deposited. Probably the nature of the lipoid deposited depends on the functional state of the organ rather than on the composition of the blood reaching it.

SUMMARY.

Observations of the plasma lipoids in the fasting state have been made in normal subjects, in patients with renal œdema, in one case of non-diabetic xanthoma, and in one case of gross diabetic lipæmia.

The normal values for the various lipoids fall within the accepted normal ranges, except in the case of glyceride, for which low values were obtained. The iodine values of the separated fatty acids normally range from 79 to 124, with an average of 105.

In lipæmia associated with renal œdema, all the lipoid fractions are increased; the glyceride forms a larger proportion of the total than normally, the free cholesterol and cholesterol ester form about the normal proportion of the total lipoids, and the phospholipin is relatively decreased. The iodine values of the separated fatty acids usually fall within the normal range. In one case in which kidney lipoids were analysed, there were extensive deposits of cholesterol ester in the kidneys in the absence of any significant increase in plasma lipoids.

The case of non-diabetic xanthoma showed a type of lipæmia similar to that found in renal œdema.

In gross diabetic lipæmia, when the glycerides form a very large proportion of the total lipoids, the iodine values of the separated fatty acids tend to be low.

In general it seems that many pathological lipæmias of moderate degree show similar changes. The lipoids in circulation probably represent a mixture of those mobilized from reserve stores and those mobilized from the liver.

I wish to express my thanks to the Honorary Physicians of the Royal Victoria Infirmary for giving me the opportunity to make observations on patients under their care, and to Dr. J. G. Thomson for the use of his post-mortem findings.

REFERENCES.

Bloor.—(1921) *J. biol. Chem.*, **49**, 201.—(1924) *Ibid.*, **59**, 543.
Boyd.—(1933) *Ibid.*, **101**, 323.
Cathcart and Cuthbertson.—(1931) *J. Physiol.*, **72**, 349.
Ellis and Maynard.—(1937) *J. biol. Chem.*, **118**, 701.
Fischer.—(1903) *Virchow's Arch.*, **172**, 30.
Gainsborough.—(1929) *Quart. J. Med.*, **23**, 101.
Gardner and Gainsborough.—(1927) *Biochem. J.*, **21**, 130.—(1934) *Ibid.*, **28**, 1631.
Hansen.—(1933) *Proc. Soc. exp. Biol.*, N.Y., **30**, 1198.
Herbert.—(1935) *Biochem. J.*, **29**, 1887.
Imrie.—(1915) *J. biol. Chem.*, **20**, 87.
Kuttner and Lichtenstein.—(1930) *Ibid.*, **86**, 671.
Lichtenstein and Epstein.—(1931) *Arch. intern. Med.*, **47**, 122.
Ling and Liu.—(1928) *Chinese J. Physiol.*, **2**, 157.
Page, Kirk and Van Slyke.—(1936) *J. clin. Invest.*, **15**, 101.
Page, Kirk, Lewis, Thompson and Van Slyke.—(1935) *J. biol. Chem.*, **111**, 613.
Sperry.—(1936) *Ibid.*, **114**, 125.
Stewart and Hendry.—(1935) *Biochem. J.*, **29**, 1683.
Stoesser.—(1936) *Proc. Soc. exp. Biol.*, N.Y., **34**, 12.
Thannhauser and Setz.—(1936) *J. biol. Chem.*, **116**, 533.
Wilson and Hansen.—(1936) *Ibid.*, **112**, 457.

THE EFFECT OF TOXÆMIA ON THE METABOLISM OF THE LIVER. DIPHTHERITIC TOXÆMIA AND CARBOHYDRATE SYNTHESIS.

M. C. A. CROSS* AND ERIC HOLMES.

From the Biochemical Laboratory, Cambridge.

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THE work described in this paper is a study of the changes which take place in the metabolism of liver-cells during diphtheritic toxæmia. Of late years so much progress has been made in the study of the biochemical events taking place in the different organs of the normal body, that it seems that a stage has been reached at which such studies can profitably be transferred to those of the organism which is suffering from the effects of pathological agents. In embarking upon such experiments we have chosen diphtheria toxin as a pathogenic agent, because it is easily obtained and standardized, is stable, and has been used before by other workers. We have studied in the first instance liver rather than any other organ, because a good deal is already known about its function, because it is known to be affected in various pathological conditions, and because it provides a plentiful supply of easily accessible material. We are, of course, aware that symptoms indicating hepatic damage are not often met with in clinical cases of diphtheria; indeed, a survey of the data suggests that, at any rate when subjected to a fairly large dose of diphtheria toxin both man, and the guinea-pig, the animal which is commonly used for standardizing diphtheria toxin, die of circulatory failure, perhaps before any severe liver damage can occur. In this connection it is probably significant that these are the two animals which are known to suffer from scurvy, that vitamin C appears to be stored in the suprarenal medulla, and that diphtheria intoxication is commonly accompanied by lesions of the supra-renal glands (Cramer, 1926). These points have been made the subject of experimental work by Thaddea (1935).

Many of the workers who have previously investigated the relationship of diphtheritic toxæmia to metabolism have employed rabbits for their experiments (Lawrence and Buckley, 1927; Corkill and Ochoa, 1934; Corkhill, 1932; Yannet and Darrow, 1933; Yannet and Goldfarb, 1933). It has been found that such toxæmic animals usually show a hypoglycæmia, but that they are very resistant to the action of insulin. Evans and Zweckwer (1926), who used as a toxic agent, not diphtheria toxin, but killed cultures of *B. coli* and *B. proteus*, were able to abolish the insulin resistance so produced by the injection of ergotamine tartrate or by section of the splanchnic nerves.

Corkill (1932) showed very clearly that diphtheria intoxication, even of short duration (24 hours), rendered rabbits resistant both to the hypoglycæmic

* Yarrow Student of Girton College.

action of insulin and to its effect on the storage of liver glycogen. Glycogen is deposited in the liver of the normal young rabbit in response to the injection of small doses either of insulin or of adrenaline. Even in early toxæmia neither of these effects can be produced. This author also found that the resistance to insulin is abolished by an injection of ergotoxine. He therefore attributes the effect of toxæmia on blood sugar to an increased outpouring of adrenaline from the suprarenals, from which organs he could demonstrate a loss of chromaffine substance. Corkill and Ochoa (1934) found that, although glycogen was still deposited in the liver of rabbits with diphtheritic toxæmia of 24 hours' duration in response to an injection of glucose, it no longer appeared after an injection of lactate. They state that at this stage of toxæmia no lesions could be found in the liver.

Yannet and Darrow (1933*b*) found that the amount of glycogen deposited in the liver of rabbits as a result of the injection either of glucose or of glucose and insulin was decreased by diphtheritic toxæmia. The duration of the toxæmia is not given in their paper. It was probably longer than 24 hours. In their earlier paper (1933*a*) they record hypoglycæmia and an increase in non-protein nitrogen and amino-acid nitrogen in the blood of severely toxæmic rabbits. The observations just discussed point to the conclusion that at any rate those disturbances of carbohydrate metabolism which occur in early toxæmia are the result of an abnormally large secretion of adrenaline.

For many years clinicians have taught that in the normal organism there is a balance of endocrine secretions, and that the so-called endocrine disorders are manifestations of a derangement of that balance in one direction or another. The experimental work of recent years is revealing the fact that the normal metabolism of carbohydrates is influenced by the anterior pituitary and probably by the suprarenal cortex, as well as by the pancreas, the thyroid, and the suprarenal medulla, and that in all probability, what we regard as normal metabolism should be looked upon as the resultant of the influence of the secretions of these different organs: and it would seem that diphtheritic toxæmia as well, probably, as other infections and intoxications (Lawrence and Buckley, 1927), upset this balance in the direction of increasing the amount of those factors (of which adrenaline may or may not be the only one) which tend to prevent the deposition of liver glycogen. A reaction of this type might well be a defensive one; toxæmia is commonly accompanied by fever, which will entail, in turn, more rapid oxidation processes; and an increased supply of carbohydrate in the blood-stream will presumably supply the tissues with oxidizable substrate, an increased consumption of which might, even so, result in a hypoglycæmia.

PLAN OF THE PRESENT WORK.

It has been our main purpose, in the present investigation, to endeavour to discover whether or not (apart from the endocrine influences to which the changes in carbohydrate metabolism in early toxæmia may reasonably be attributed) there were any alterations in the metabolism of the liver-cells themselves. We should expect such to be the case, since in the later stages of toxæmia lesions in the liver can be demonstrated by histological methods.

Slices of liver tissue, shaken at 38° C. in a bicarbonate-buffered Ringer's solution in an $O_2 + CO_2$ atmosphere, will synthesize yeast-fermentable carbohydrate from non-carbohydrate substances (Gemmill and Holmes, 1936; Bach and Holmes, 1937). They will, in fact, also synthesize glycogen. By comparing the results obtained when tissue from normal and toxæmic animals respectively was thus shaken in the presence of different substrates, it has been possible to show that the power of carbohydrate synthesis is affected by the toxæmia. Observations have also been made on the respiratory quotient and oxygen consumption of the slices.

When slices of liver tissue are removed from the animal and shaken *in vitro*, it is obvious that the tissues are cut off from such supplies of hormone as may be supposed to reach them continually by the blood-stream under normal circumstances. But it is not certain that they may not carry with them supplies of one or more hormones sufficient to affect their behaviour during the time of the experiment. These points can be cleared up only by experiments performed on tissues from animals from which the various endocrine organs have been removed, or upon slices to which the hormones have been added *in vitro*. Experiments of this kind are at present being performed, and until they have been completed, it will be impossible to say with certainty that the effects which we have observed are due solely to the influence of the toxin upon the liver-cells. But our results have been quite definite, and it seems desirable to publish them at this stage.

At the beginning of this investigation guinea-pigs were employed. As, however, most other investigators had used rabbits, and as, in our experiments, the guinea-pigs appeared to die from circulatory failure without any marked histological lesions appearing in the liver, we thought it better, after a short time, to confine our experiments to rabbits. Young animals (500–1200 g.) were always used.

For the purpose of demonstrating carbohydrate synthesis in liver slices, the animals must be starved for at least 24 hours before being killed, for if the initial carbohydrate content of the slice is high, little or no additional carbohydrate seems to be formed during the period of the experiment. The toxæmic animals, in any case, usually refuse food after 24 hours have elapsed from the time of the injection. We therefore made a practice of removing all food (but not water) from the cage 24 hours before the beginning of the experiment.

We are indebted to the kindness of Mr. A. T. Glenny, of the Wellcome Institute for Physiological Research, for the sample of diphtheria toxin which we have used. We have found that subcutaneous injection of 4 guinea-pig minimal lethal doses per kg. of rabbit kills the animals in from 50 to 70 hours, and this dose was used consistently throughout our experiments.

The technique (essentially that of Cori and Cori, 1933) employed for the estimation of total fermentable carbohydrate has recently been described in detail (Bach and Holmes, 1937). Glycogen was determined by the method of Good, Cramer and Somogyi (1933), the details of the procedure being as follows:

The cups contained 2 ml. of Ringer's solution (not 3 ml., as in the experiments in which total carbohydrate was determined). While they were still in the thermostat, small glass funnels

were attached to the gas outlet tubes by short pieces of pressure tubing, and into each funnel was pipetted 1·0 ml. of 80 p.c. KOH solution. This could be done with no more than a momentary interruption of the shaking. At the required moment the tap of the gas outlet tube was opened, and the manometer tap was also opened to the atmosphere. The potash ran into the cup, and a few moments' shaking mixed it with the tissue, giving about a 20 p.c. concentration of potash in the cup. The cups were then removed, and dipped into a boiling water bath and gently shaken until the tissue had disintegrated. The contents of the cups were then transferred to centrifuge tubes, the cups being washed out with 1 c.c. of hot water. The tubes were heated for 30–40 minutes on a boiling water-bath, and the precipitation with hot alcohol and subsequent manipulations carried out. The sugar was estimated after hydrolysis, by the method of Hagedorn and Jensen (1922). Unless otherwise indicated, the "initial" samples, both for total carbohydrate and for glycogen, were fixed by the addition of acid or of potash respectively immediately after the cups had been filled with gas mixture, *i. e.* after they had been shaken in the thermostat for 10 minutes.

THE EFFECT OF TOXÆMIA ON THE POWER OF THE LIVER TISSUE TO SYNTHESIZE CARBOHYDRATE FROM VARIOUS SUBSTANCES.

Bach and Holmes (1937) have shown that slices of rat liver will synthesize carbohydrate from lactate, pyruvate and alanine. In addition to these substances we have tested the following: glycerol, *α*-glycerophosphate, glyceric aldehyde, butyrate, acetate, glyceric acid and succinic acid. All, except butyrate, acetate and glyceric acid, have been shown by previous workers to give rise to carbohydrate formation both in the liver of the intact animal, and also when perfused through a surviving liver. While our work was in progress, Cori and Shine (1936) showed that normal liver slices formed glucose from the following substances: fructose, mannose, galactose, glycerol, *α*-glycerophosphate, *β*-glycerophosphate, glyceric aldehyde and dihydroxyacetone.

It was our purpose, in the first instance, to compare the capacity of the liver in different stages of toxæmia with that of the normal liver to form carbohydrate from different substrates. Previous workers had used the glycogen content of the liver at the time of death for this purpose; but we feel that the tissue-slice technique affords a better opportunity of studying the functional capacity of the liver-cells; for the glycogen content of the liver in the intact animal at any given moment must obviously depend on the interaction of many divers factors, such as the secretion of hormones, the rate of blood-supply, the oxygenation of the blood reaching the liver, and, perhaps, other constituents of the blood. (We find, for instance, that after 48 hours' toxæmia the blood urea of rabbits is commonly much increased.) The results of a large number of experiments are given in Table I and Table II. The liver slices were always shaken at 38° C. in Ringer's solution containing 0·025 *M* sodium bicarbonate in equilibrium with an atmosphere containing 5 p.c. CO_2 and 95 p.c. oxygen. All acid substances were, of course, neutralized with soda before addition to the Ringer. The duration of the experiments was, in all cases, 2 hours.

An inspection of Table I shows that all the substances added, except sodium acetate, gave rise to a greater or less increase of fermentable carbohydrate under the conditions of the experiment. There is also an increase of fermentable carbohydrate in the absence of any added substrate. Bach and

TABLE I.—*Carbohydrate Formation in Slices of Liver Tissue from Starved but otherwise Normal Rabbits.*

Date.	Initial value.	Increase.										
		Without added substrate.	With lactate.	With pyruvate.	With alanine.	With glycerol.	With glycero-phosphate.	With glyceric aldehyde.	With glycerate.	With acetate.	With succinate.	With butyrate.
20.i.36	9·67	2·67	6·28	..	..	..	..	..	..	..	..	..
15.ii.36	9·21	1·54	8·15	..	..	..	..	..	..	..	..	..
26.ii.36	9·26	1·90	4·45	..	..	..	..	..	..	..	..	..
29.ii.36	3·69	1·47	4·37	..	..	..	..	..	..	..	..	..
16.iii.36	7·71	2·47	4·25	..	..	..	..	..	..	..	..	..
19.iii.36	5·70	1·11	2·63	..	..	..	..	..	..	..	..	..
24.vii.36	2·17	2·40	3·93	..	..	..	..	..	..	..	..	..
31.vii.36	1·75	2·38	3·35	..	..	..	..	..	..	..	..	..
3.viii.36	2·07	1·37	3·50	..	..	..	..	..	..	..	..	..
5.viii.36	2·38	0·79	5·38	..	..	..	..	..	..	..	..	..
6.viii.36	2·33	1·84	5·86	..	..	..	..	..	..	..	..	..
8.viii.36	6·44	1·92	4·18	..	..	..	..	..	..	..	..	..
10.viii.36	2·40	3·87	5·81	..	..	..	..	..	..	..	..	..
12.viii.36	4·16	0·54	1·35	..	..	..	..	..	..	..	..	..
15.ix.36	3·70	2·04	..	..	3·24	..	..	..	..	..	..	..
28.ix.36	8·22	1·19	..	..	2·78	..	..	..	..	..	..	..
30.ix.36	2·05	4·10	5·26	..	2·52	..	..	..	..	..	..	..
11.x.36	4·19	1·00	*3·68	..	..	..	..	..	..	..	..	..
21.x.36	7·33	1·47	*1·94	..	..	..	..	..	..	..	..	..
24.x.36	7·06	1·17	*3·54	..	..	..	..	..	..	..	..	..
28.x.36	2·56	1·11	..	..	..	..	3·84	..	..	..	..	..
5.xi.36	4·16	1·79	2·34	..	..	..	..	..	..	..	..	..
18.xi.36	3·52	3·43	4·96	..	..	..	..	..	..	..	..	..
24.xi.36	2·33	2·01	*4·40	..	..	..	..	..	..	..	..	..
27.xi.36	3·36	1·33	*4·93	..	..	9·78	6·80	..	..	..	..	..
7.xii.36	3·25	1·54	..	..	..	..	..	†4·47	..	..	..	..
10.xii.36	3·10	2·45	..	..	..	..	..	†7·46	..	..	..	..
14.xii.36	12·13	2·92	..	..	..	..	..	7·87	..	..	..	..
17.xii.36	2·84	1·36	..	..	..	..	..	4·92	..	1·74	..	2·76
21.xii.36	8·34	1·80	..	..	..	..	..	..	..	2·34	..	3·03
13.i.37	2·50	2·43	7·84	..	3·87	5·82	..	..	..	..	..	

TABLE I—*continued.*

Date.	Initial value.	Increase.										
		Without added substrate.	With lactate.	With pyruvate.	With alanine.	With glycerol.	With glycero-phosphate.	With glyceric aldehyde.	With glycerate.	With acetate.	With succinate.	With butyrate.
15.i.37 . .	6·13	4·22	..	..	2·19	..	..	..	..	..	..	..
27.i.37 . .	10·11	0·81	..	7·26	5·34	8·67	..	..	..	..	..	..
1.ii.37 . .	4·64	3·06	..	6·95	2·50	7·80	..	..	..	..	..	..
3.ii.37 . .	4·56	1·51	..	6·13	3·12	5·83	..	7·67	..	..	..	2·65
29.iv.37 . .	4·45	1·49	..	..	..	6·70	..	..	..	..	..	2·75
3.v.37 . .	6·50	3·32	..	..	..	6·90	..	..	..	..	..	5·02
5.v.37 . .	3·79	1·67	..	..	..	5·47	..	..	..	..	..	3·27
7.v.37 . .	9·33	2·57	..	..	..	7·76	3·86	..	..	..	..	4·43
12.v.37 . .	2·92	1·76	..	..	..	..	4·02	..	..	2·27	..	..
14.v.37 . .	3·62	2·30	..	6·25	..	..	4·80	8·14	..	1·58	..	..
1.vi.37 . .	5·84	3·34	..	..	..	..	..	8·56	1·98	..	..	..
3.vi.37 . .	5·24	2·21	..	..	..	..	..	..	3·17	..	2·78	..
8.vi.37 . .	3·41	1·78	..	..	..	..	..	8·92	..	..	..	..
15.vi.37 . .	4·90	3·30	..	..	..	..	..	..	4·42	..	3·84	..
18.vi.37 . .	3·99	2·19	..	..	..	..	..	..	4·54	..	2·90	..
24.vi.37 . .	2·87	2·62	..	..	..	..	..	..	4·20	..	3·38	..
Average value .	4·93	2·05	4·44	6·57	3·59	7·19	4·51	7·25	3·64	1·98	3·22	3·41
Increase due to substrate added	..	..	2·39	3·52	1·54	5·14	2·46	5·20	1·59	−0·07	1·17	1·36

All quantities mg. per g. of fresh tissue.
* 0·2 p.c. lactate. Remainder 0·4 p.c. lactate.
† 0·18 p.c. glyceric aldehyde. Remainder 0·36 p.c. glyceric aldehyde.

TABLE II.—*Carbohydrate Synthesis in Slices of Liver Tissue from Animals Injected with Diphtheria Toxin.*

Date.	Treatment.	Initial.	Without added substrate.	With lactate.	With pyruvate.	With alanine.	With glycerol.	With α-glycero-phosphate.	With glyceric aldehyde.	With butyrate.
24.iii.36	24 hours' toxæmia	1·85	1·19	4·19	..	..	..	..	..	..
26.iii.36	„ „	3·83	0·98	3·53	..	..	..	..	..	..
28.iii.36	„ „	3·12	1·65	3·51	..	..	..	..	..	..
1.vii.36	„ „	7·36	1·12	4·30	..	..	..	..	..	..
3.iii.37	„ „	4·38	2·90	5·90	..	..	6·94	..	..	..
8.iii.37	„ „	2·27	2·18	3·75	..	..	..	..	..	..
10.iii.37	„ „	4·50	1·94	7·40	..	..	..	..	..	..
7.vi.37	„ „	2·93	2·18	3·74	..	..	..	..	..	..
10.vi.37	„ „	3·64	2·55	..	2·87	..	..	..	..	..
20.vi.37	„ „	6·97	1·20	..	3·54	..	..	..	..	..
Average value for 24 hours' toxæmia		4·10	1·79	4·54	..	..	..	..	..	..
Increase due to substrate added		..	..	2·75	..	..	..	..	..	..
16.iv.36	36 hours' toxæmia	2·66	0·66	0·82	..	..	..	..	..	..
20.iv.36	43 „ „	2·71	1·80	0·83	..	..	..	..	..	..
22.iv.36	40 „ „	3·55	1·36	3·41	..	..	..	..	..	..
6.v.36	42 „ „	4·27	2·35	5·27	..	..	..	..	..	..
7.v.36	44 „ „	2·63	1·30	1·56	..	..	..	..	..	..
24.v.37	38 „ „	7·15	2·55	..	2·83	..	..	..	..	2·24
26.v.37	38 „ „	3·76	1·49	..	0·93	..	..	..	2·33	1·24
28.v.37	38 „ „	3·36	2·65	..	2·44	..	..	..	6·06	2·52
Average value for 36–44 hours' toxæmia		3·76	1·77	2·38	2·06	..	..	..	..	2·00
Increase due to substrate added		..	..	0·61	0·29	..	..	..	..	0·23

TABLE II—*continued*.

Date.	Treatment.	Initial.	Without added substrate.	With lactate.	With pyruvate.	With alanine.	With glycerol.	With α-glycero-phosphate.	With glyceric aldehyde.	With butyrate.
25.i.36	48 hours' toxæmia	4·37	0·22	0·72	..	..	..	..	..	..
29.i.36	48 „ „	4·47	0·87	2·16	..	..	..	..	..	..
11.v.36	48 „ „	1·12	0·33	0·50	..	..	..	..	..	..
18.v.36	48 „ „	3·30	2·57	2·69	..	..	..	..	..	..
20.v.36	47 „ „	4·17	1·76	4·03	..	..	..	..	..	..
25.v.36	48 „ „	1·32	1·66	1·98	..	..	..	..	..	..
1.vi.36	48 „ „	4·61	2·61	3·14	..	..	..	..	..	..
9.vii.36	50 „ „	2·95	0·41	1·15	..	..	..	..	..	..
17.vii.36	49 „ „	2·87	1·84	2·03	..	..	..	..	..	..
22.vii.36	49 „ „	2·44	2·13	1·31	..	..	..	..	..	..
16.ix.36	48 „ „	1·49	1·01	..	..	0·92	..	..	..	..
18.ix.36	48 „ „	3·64	2·48	3·35	..	2·51	..	..	..	..
23.ix.36	48 „ „	1·02	0·24	0·45	..	0·68	..	..	..	..
2.x.36	48 „ „	3·55	1·78	3·81	..	2·81	..	..	..	..
5.x.36	48 „ „	1·29	2·25	2·56	..	2·79	..	..	..	..
9.x.36	48 „ „	1·42	1·33	0·82	..	1·21	..	..	..	..
14.x.36	48 „ „	1·98	1·84	1·05	2·44	..	..	..	..	..
16.x.36	48 „ „	4·06	2·07	1·84	2·49	..	..	..	..	..
19.x.36	48 „ „	2·46	1·75	2·52	2·32	..	..	..	..	..
30.x.36	48 „ „	2·34	1·92	3·21	..	..	..	3.55	..	..
2.xi.36	48 „ „	3·12	2·52	..	..	..	..	4.90	..	..
6.xi.36	48 „ „	2·96	1·09	2·84	..	..	..	2.97	..	..
9.xi.36	48 „ „	1·54	0·57	1·37	..	..	..	2.70	..	..
11.xi.36	53 „ „	2·08	2·64	3·49	..	..	..	4.59	..	..
13.xi.36	48 „ „	1·99	1·20	1·76	..	..	2·75	2.75	..	..
30.xi.36	48 „ „	6·47	2·27	3·30	..	..	..	4.52	..	..
12.i.37	48 „ „	1·99	1·60	2·48	..	..	3·90	..	6·55	1·32
19.i.37	51 „ „	1·50	1·27	1·90	..	..	2·56	..	3·14	..
21.i.37	52 „ „	1·31	2·77	4·67	3·37	4·04	6·00	..	7·22	..
25.i.37	Moribund	1·21	1·11	0·92	1·09	0·79	..	..	4·24	..
Average value for 48–52 hours' toxæmia		2·64	1·57	2·22	2·34	1·97	3·80	3·71	5·29	..
Increase due to substrate added		..	..	0·65	0·77	0·40	2·23	2·14	3·72	..

Holmes (1937) have shown that a part of this increase is abolished by insulin, and they have given reasons for believing that, in so far as such is the case, it represents synthesis from amino-acids. For the rest, it may be remarked that, when the respiratory quotient of slices of liver from a starved animal are shaken in a Dixon-Keilin (Dixon, 1934) manometric apparatus, the respiratory quotient is so low that it suggests the synthesis of carbohydrate from fat. In this connection it is of great interest that there is a small but distinct increase in fermentable carbohydrate in the presence of added butyrate.

The metabolic events going forward even in an isolated liver slice are probably very complex, and it cannot be assumed that, because the addition of some particular substance gives rise to an increase in fermentable carbohydrate, that substance has necessarily itself been converted into carbohydrate. Such may have been the case, but until further evidence (such as might be afforded by a study of the R.Q. of the slices in the presence of the different substrates) has been obtained, the possibility remains that the added substrate has merely increased the rate at which other materials, already present in the tissue, are converted into carbohydrate.

Table I shows that fermentable carbohydrate is increased most by glycerol and glyceric aldehyde, either of which cause an increase more than twice as great as that which occurs in the absence of substrate. The remaining substances stand in the following order: pyruvate, *a*-glycerophosphate, lactate, glycerate, alanine, butyrate, succinate; while in the presence of acetate there is no extra synthesis.

In Table II are arranged the values for the increases in fermentable carbohydrate observed after the addition of some of these same substrates to the livers of animals which had been injected with diphtheria toxin. The experiments are arranged in three groups: the first, animals with toxæmia of 24 hours' duration, the second, animals with toxæmia of from 36–44 hours' duration, and the third, with toxæmia of 47 hours' duration and over.

It will be seen that, after 24 hours' toxæmia, the power of the slices to synthesize carbohydrate, either in the absence of any added substrate or in the presence of added lactate, is hardly at all impaired.

In the second group, however, there is distinctly less synthesis in the presence of lactate and pyruvate; moreover, the " initial " values are distinctly lower than the corresponding figures for normal starved animals. This applies with still greater force to the third group, which was the only one in which we have investigated the effect of the addition of substrates other than lactate, save for pyruvate in a few cases in Groups I and II, and butyrate and glyceric aldehyde in Group II. The figures show that, while the synthesis in the presence of all the added substances is affected, it is affected by no means uniformly in each case. The synthesis in the presence of lactate, pyruvate, alanine and butyrate is greatly diminished, and to about the same extent in each case. Since it is probable that all the first three substances pass through a common stage (alanine, on oxidative deamination will give pyruvic acid, and pyruvic acid may be reduced to lactic acid or lactic acid oxidized to pyruvic), we should expect them to behave alike. The synthesis from glycerol is affected less than that from the above mentioned substances, but still very considerably, and far more than that from glyceric aldehyde or from

glycerophosphate. We have at present no experimental evidence which we can bring forward to explain this particular point.

For purposes of comparison, the increase, over and above that occurring in the absence of substrate which is observed in the toxæmic animals, is shown below as a percentage of the similar increase observed in the normal ones.

Substrate.	Percentage of synthetic power remaining after 48 hours' toxæmia.
None	77
Lactate	27
Pyruvate	22
Alanine	26
Glycerol	43
Glycerophosphate	87
Glyceric aldehyde	72
Butyrate	17

DISAPPEARANCE OF LACTIC ACID ADDED TO THE SLICES.

Estimations of lactic acid, carried out on the initial and final samples of tissue to which lactate has been added, show clearly that the power of the tissue from toxæmic animals to remove lactate is diminished. This observation is in accordance with the findings of Minot (1931) and Minot, Dodd and Saunders (1934) that in poisoning by chloroform and by guanidine in dogs (both of which substances damage the liver) there is an increase of lactic acid in blood and urine. Our estimations were performed by the method of Friedmann, Cotonio and Shaffer (1927).

The results are given in Table III.

TABLE III.—*Lactic Acid in mg. at the Beginning and End of 2-Hour Experiments in which Liver Slices were shaken in 3 ml. of Bicarbonate + CO_2 buffered Ringer's Solution containing 0·2 p.c. Lactic Acid as Sodium Lactate. Toxæmia of 48 hours' duration.*

The signs − and + denote respectively the disappearance and appearance of lactic acid.

Normal.			Toxæmic.		
Initial.	Final.	Difference.	Initial.	Final.	Difference.
6·61	4·56	−2·05	6·61	6·73	+0·12
6·03	3·27	−2·76	6·55	5·78	−0·77
6·41	4·50	−1·91	8·08	7·83	−0·25
7·31	4·57	−2·74	6·81	5·82	−0·99
..	..	..	5·43	5·62	+0·19
..	..	..	6·10	5·86	−0·24
	Average	−2·36		Average	−0·32

The average quantity of tissue used in the experiments with normal animals was 0·76 g. The disappearance of lactic acid per g. of tissue was thus 3·12 mg.

In the experiments with toxæmic animals the average quantity of tissue

was 0·96 g., and the disappearance of lactic acid per g. of tissue 0·33 mg. Reference to Tables I and II shows that the corresponding synthesis of carbohydrate was 2·39 mg./g. and 0·65 mg./g. respectively.

EFFECT OF INSULIN ON SLICES OF LIVER FROM TOXÆMIC ANIMALS.

It is of some interest that toxæmia abolishes the power of insulin to inhibit gluconeogenesis. Experiments on this point have been carried out only in the absence of added substrate. The results are shown in Table IV.

TABLE IV.—*Increase in Fermentable Carbohydrate in the Absence of Added Substrate, With and Without Insulin.*

	Normal.		Toxæmic.	
	No insulin.	Insulin.	No insulin.	Insulin.
	2·01	1·02	0·24	0·28
	2·43	1·42	2·25	2·06
	4·10	3·47	1·33	1·10
	2·00	1·02	1·84	2·05
	..	..	2·07	2·24
	..	..	1·75	2·26
Averages	2·635	1·707	1·580	1·665
	Decrease with insulin, 0·928.		Increase with insulin, 0·08.	

This observation is clearly (in the light of the results of Bach and Holmes) compatible with the hypothesis that toxæmia reduces the synthesis of carbohydrate from certain amino-acids, of which we suppose the synthesis from alanine to be typical. It may also, in part, explain the " insulin resistance ", at any rate in the later stages of toxæmia, observed by other workers.

It seems clear, from these experiments, that the power of the liver-cells to synthesize carbohydrate is not seriously impaired for the first 24 hours of toxæmia, but that a failure in gluconeogenesis subsequently appears, which, however, does not affect the power to synthesize carbohydrate from all substrates to an equal degree. Naturally, we should tentatively draw the conclusion, either that the pathway by which α-glycerophosphate and glyceric aldehyde are converted to carbohydrate is different from that followed by lactate, pyruvate and alanine, or that in the case of the latter substances one or more extra steps are involved.

Corkill and Ochoa (1934) have shown that the glycogen content of the liver is much diminished, and the power to lay down glycogen in response to an injection of lactate apparently disappears, in rabbits with diphtheria intoxication of not more than 24 hours' duration. Yannet and Darrow (1933*b*) had previously shown (probably using a longer period of toxæmia) that the power to deposit liver glycogen from glucose was deficient.

We have repeated the experiments of Corkill and Ochoa and confirm their results, except in one particular. They state that in early toxæmia the power to deposit glycogen in response to an injection of glucose is retained. We find that, while this power is indeed still present, it is definitely diminished. Our results are shown in Table V.

TABLE V.—*Glycogen Content of Liver Immediately after Death.*

Date.	Normal starved rabbits. Injection.	Initial.	Final.	Starved rabbits 24 hours after injection of toxin. Injection.	Initial.	Final.
28.v.36	10 ml. 20 p.c. lactate	0·06	1·21			
8.vi.36	10 „ 10 „ „	0·18	0·81	10 ml. 10 p.c. lactate	0·13	0·03
10.vi.36	20 „ 5 „ „	0·25	1·70	16 „ 6·25 „ „	0·17	0·03
8.vii.36	20 „ 5 „ „	0·34	1·56	20 „ 5 „ „	0·07	0·08
29.vii.36	20 „ 5 „ „	0·60	1·03	20 „ 5 „ „	0·02	0·02
18.vi.36	8 „ 25 „ glucose	0·04	5·39	8 „ 25 „ glucose	0·06	3·36
26.vi.36	8 „ 25 „ „	0·03	2·61	8 „ 25 „ „	0·08	0·70
3.vii.36	8 „ 25 „ „	..	3·77	8 „ 25 „ „	0·04	0·52

Glycogen in g. per 100 g. fresh tissue. All rabbits used on the same day belong to the same litter. Two hours elapsed between the injection of glucose or lactate and the killing of the animal for "final" values. The animals for "initial" value were themselves, of course, not injected, but were killed when their litter mates were injected. Racemic lactic acid was used for injection.

It seems clear from our experiments that the failure in early toxæmia is not in the power to manufacture carbohydrate, but in the power to convert it into, or retain it as, glycogen. Glucose can be converted into glycogen by the liver; but since glucose is not an intermediate step in the breakdown of glycogen in muscle, it is at least possible that glycogen can also be synthesized in the liver without the intermediate formation of glucose. Toxæmia, therefore, may act by accelerating the breakdown of glycogen, rather than by preventing its synthesis. After an injection of glucose or fructose the concentration of hexose may be sufficiently high, even in toxæmia, to push the reaction (glycogen $\rightleftharpoons$ glucose) from right to left; but if the breakdown of glycogen is accelerated by toxæmia, the rate of gluconeogenesis, even though nearly as great as in the normal animal, may never be sufficient to cause the accumulation of glycogen.

Since there is so much evidence to suggest that during toxæmia there is an excessive liberation of adrenaline into the circulation, it occurred to us to try whether or no we could demonstrate a synthesis of glycogen *in vitro* in slices of normal liver, and if so, whether this synthesis occurred in slices of liver from toxæmic animals; and also whether in the normal animal it was affected by the addition of adrenaline.

In view of the fact that liver glycogen breaks down very rapidly when the tissue is deprived of oxygen, we hardly expected to succeed in demonstrating glycogen synthesis in liver slices *in vitro*, where, after all, the conditions to which the tissue are subjected are far from normal. The synthesis which does occur *in vitro* is small, in most cases much smaller than that which takes place in the livers of intact animals in a corresponding time. It must be confessed, however, that our surprise was rather that it happened at all than that it was less extensive than it is in the intact animal. It should be emphasized that, for glycogen synthesis to be demonstrated, the potash must be added to the tissue while it is still well aërated. If the shaker is stopped, and the cups removed from the bath before the tissue is fixed, the glycogen may have broken down again.

Table VII shows the figures obtained for the synthesis of glycogen in the presence of various substrates in slices of normal livers, in slices of livers from

animals injected with toxin 24 hours previously (at which time it will be remembered the formation of fermentable carbohydrate is almost unimpaired), and in normal livers to which adrenaline was added. The adrenaline (base) was dissolved in the amount of dilute hydrochloric acid appropriate to neutralize it, and 0·1 c.c. of a solution containing the required amount of adrenaline was placed in a Keilin pot and tipped as soon as the filling of the cups with gas mixture was complete. In view of the fact that adrenaline is inactivated by slices of rat's liver when shaken in oxygen (Blaschko and Schlossmann, 1936), we were prepared to find that this substance was without effect in our experiments. We suppose that it is, in fact, extremely potent in accelerating glycogen breakdown, and that although of the small amounts which we added much was destroyed, yet sufficient remained to exert its effect. Possibly, too, rabbit liver is less active than rat liver in oxidizing adrenaline.

The following experiment (Table VI) was designed to show the limits of concentration of adrenaline necessary to prevent the appearance of glycogen in liver slices shaken with 0·4 p.c. glucose. The duration of the experiment was 2 hours. The amount of adrenaline is given in γ per c.c. of fluid in which the slices were suspended.

TABLE VI.

Conc. of adrenaline, γ per c.c.	250	125	62	25	0
Glycogen, mg. per g. of tissue	0·53	0·66	0·86	1·82	1·80

The initial glycogen content of the tissue was, in this case, determined by putting the slices into potash directly after weighing and was 1·13 mg. per g. It is evident that the minimum effective concentration of adrenaline lies between 62 and 25 γ per c.c. The amount added (500 γ in 2 ml.) in the experiments quoted in Table VII was, therefore, ample to produce the maximum effect.

Table VII illustrates several points of interest. As might be expected, the synthesis of glycogen *in vitro* is greater in the presence of glucose than in that of lactate; pyruvate seems to be rather more effective than lactate (*cf.* Table I). By far the greatest synthesis, however, occurs in the presence of fructose.

Adrenaline cuts down the synthesis from both glucose and lactate *in vitro*, but it cannot be said, on the present evidence, to affect the synthesis from lactate more than that from glucose. On the other hand, the effect of toxæmia, *in vitro* as *in vivo*, is to reduce the synthesis from lactate more than that from glucose. As is indicated in Table VII, reference to Table II shows that these tissues were still forming carbohydrate though they could not deposit glycogen. It is not, therefore, by any means clear that the effects observed in toxæmia can be referred to the liberation of adrenaline.

It is a fair summary of our evidence to say that we have found that, as toxæmia progresses, there is a progressive failure of the power of the liver to form carbohydrate; but also that, before this becomes clearly recognizable, there is a failure to deposit glycogen, except when the concentration of glucose is kept very high, as it is by adding glucose to the slices or by injecting glucose

TABLE VII.—*Synthesis of Glycogen by Slices of Liver*

TABLE VII.—*Synthesis of Glycogen by Slices of Liver.*

Normal, starved rabbits.							Toxæmic rabbits (24 hours' toxæmia).				
		Increase above initial.							Increase above initial.		
Date.	Initial.	Without added substrate.	With lactate.	With glucose.	With lactate + adrenalin.	With glucose + adrenalin.	Date.	Initial.	Without added substrate.	With lactate.	With glucose.
1.ii.37	0·77	..	..	0·73	..	..	25.ii.37	0·60	0·08	0·05	1·15
3.ii.37	0·45	0·53	..	1·63	..	..	3.iii.37*	0·52	0·34	1·94	3·35
10.ii.37	1·41	0·58	0·11	..	..	..	8.iii.37*	0·24	0·42	0·50	1·56
13.ii.37	1·27	0·20	0·19	..	..	..	10.iii.37*	0·38	0·04	0·10	0·62
15.ii.37	0·45	0·34	1·47	..	..	..	7.vi.37*	0·53	—0·04	0·25	0·59
18.ii.37	0·39	0·11	0·86	..	..	..	10.vi.37*	0·32	0·51	0·72	1·84
1.iii.37	0·44	0·15	1·25	..	..	..	25.vi.37*	0·62	0·24	..	1·15
5.iii.37	0·43	0·13	0·70	..	..	..	..	..	..	..	..
13.iii.37	0·38	0·14	1·15	1·60	0·63	0·92	..	..	..	..	..
16.iii.37	0·42	0·04	0·25	0·24	0·02	0·10	..	..	..	..	..
18.iii.37	0·38	—0·02	0·45	0·79	0·04	0·04	..	..	..	..	..
22.iii.37	0·40	0·30	1·46	2·81	0·28	0·48	..	..	..	..	..
24.iii.37	3·80	—0·08	2·24	4·57	..	..	..	..	..	..	..
27.iii.37	1·13	..	..	0·67	..	..	..	..	..	..	..
30.iii.37	0·82	..	..	0·94	..	—0·14	..	..	..	..	..
23.iv.37	0·35	..	0·32	..	..	..	..	..	..	..	..
21.iv.37	0·34	..	0·39	..	..	..	..	..	..	..	..
18.v.37	0·57	0·71	..	..	..	..	..	..	..	..	..
1.vi.37	0·46	0·32	..	..	..	..	..	..	..	..	..
21.v.37	3·11	0·43	..	..	..	..	..	..	..	..	..
3.vi.37	0·44	0·44	..	..	..	..	..	..	..	..	..
16.vi.37	0·55	—0·01	0·96	2·72	..	..	..	..	..	..	..
21.vi.37	0·97	—0·27	0·45	..	..	..	..	..	..	..	..
Average	0·86	0·22	0·82	1·67	0·24	0·28		0·46	0·23	0·59	1·46
Increase due to added substrate			0·60	1·45	0·02	0·06		..	..	0·36	1·23

* Figures for total fermentable carbohydrate also available. See Table II, under appropriate dates.

The following additional results may be summarized :

Normal animals : Increase with fructose (average of 6 experiments) 4·48 mg./g.
,, ,, ,, ,, pyruvate (,, 7 ,,) 1·03 ,,
Toxæmic ,, ,, ,, ,, (,, 3 ,,) 0·47 ,,

All quantities mg./g. fresh tissue. Concentration of substrates, 0·4 p.c. Duration of experiments, 2 hours. Amount of adrenaline added, 0·5 mg.

into the animal. Corkhill and Ochoa (1932) were inclined to interpret their results as indicating a failure in carbohydrate formation in animals in early toxæmia. The present experiments, however, do not support such a view, although they do show that failure of the power to form carbohydrate appears at a later stage.

It would be of great interest if we could succeed in disentangling the effects of toxæmia upon the biochemical machinery of the liver-cells themselves from effects which may be secondary to damage to endocrine glands such as the suprarenal cortex or medulla, the pituitary, or the pancreas. It is obvious that when we work with isolated tissues, we cut them off from the continuous supply of hormones which, presumably, they receive during life. But we do not know how much of these substances they may possess at the beginning, or retain during the course of the experiment. We have found, however, that they are sensitive to adrenaline, at least, in very low concentration.

HISTOLOGICAL CHANGES.

Frozen sections of the livers of all the toxæmic animals were cut immediately after death and stained for fat with Scharlach R. Other portions were embedded in paraffin, cut, and stained with hæmatoxylin and van Gieson's stain. Especially in early toxæmia, the liver shows fatty infiltration, though so also do the livers of animals which have merely been starved. The paraffin sections show progressive pathological change, amounting in the early stages to a few areas of focal necrosis, and, in the later stages, to very extensive necrosis. It is proposed to publish separately an account of the histology of these tissues. It is at present difficult to say how far the histological changes can be correlated with the biochemical findings.

RESPIRATORY METABOLISM.

Besides investigating the power of the liver slices to form carbohydrate, we have examined their respiratory metabolism by the method of Dixon and Keilin (Dixon, 1934). This study is so far incomplete, in that we have not yet sufficiently investigated all the substrates used in the carbohydrate experiments. A summary of some of the figures available, however, may usefully be given here. The matter will be dealt with in more detail in a later communication.

Information can be obtained from an experiment with the Dixon-Keilin apparatus on the following points :

(1) The oxygen consumption of the tissue.

(2) The true respiratory quotient, *i. e.* $\frac{\text{respiratory } CO_2}{\text{oxygen absorbed.}}$

(3) A figure representing what is commonly called " glycolysis ".

This last is actually the amount of CO_2 absorbed or liberated in consequence of the production of base or acid respectively in the medium during the course of the experiment, as opposed to that produced by respiratory processes. It is obvious that the full deductions cannot be drawn from this figure until it is known what bases or acids are concerned, and whether there be one or more

than one. For example, lactic acid may be burned or converted into carbohydrate by the slices, thus liberating base. This would cause the absorption of CO_2 (" negative glycolysis "); but if at the same time aceto-acetic acid is being produced from neutral fat, that process will cause a positive glycolysis which may partially or wholly mask the negative glycolysis due to the removal of lactic acid.

Until, therefore, the events occurring in the tissue can be analysed in greater detail, the interpretation of the figures for " glycolysis " must remain, partly at least, obscure.

A summary of some of the experiments already performed is given below :

Table VIII.—Respiratory Metabolism of Slices of Liver from Normal and Toxæmic Animals. Quantities in μl. of gas at N.T.P. per g. of fresh tissue per hour. Concentration of substrates 0·4 p.c.

The number in brackets indicates the number of experiments upon which the figure is based; " + " indicates the evolution, " − " the absorption of gas.

	O_2 Respired.	R.Q.	Glycolysis.
Normal, no added substrate	−1841 (16)	0·59 (16)	+130 (10)
Toxæmic, 24 hours, no added substrate	−1440 (4)	0·500 (4)	+194 (4)
Toxæmic, 48 hours	−1408 (14)	0·502 (14)	+103 (14)
Normal, with lactate 0·4 p.c.	−2230 (4)	0·65 (4)	−558 (4)
Toxæmic 24 hours, with lactate 0·4 p.c.	−1607 (4)	0·59 (4)	−296 (4)
Toxæmic 48 hours with lactate 0·4 p.c.	−1325 (9)	0·49 (9)	−269 (9)
Normal with pyruvate 0·4 p.c.	−2492	1·36	−1682
" " " "	−2305	1·25	−1396
" " " "	−1890	1·34	−1061
Average of experiments with pyruvate only	−2229	1·316	−1379
Toxæmic 24 hours, with pyruvate 0·4 p.c.	−1415	1·14	−871
Ditto	−1596	0·68	−410
"	−2505	1·14	−1400
Average of experiments with pyruvate only	−1838	0·986	−983
Toxæmic 38 hours, with pyruvate 0·4 p.c.	−1530	0·86	−329
Ditto	−1295	0·89	−274
"	−1832	0·74	−271
Average	−1552	0·83	−291

It will be observed that toxæmia reduces the oxygen consumption and the respiratory quotient both of the tissue alone and of the tissue with lactate or pyruvate. The low R.Q. of the normal tissue is due to the fact that the animals are starved (Gemill and Holmes, 1936). The still lower R.Q. in toxæmia would be compatible with a diminution of the power to oxidize carbohydrate, and a shift of the metabolism even more in the direction of fat usage than is already brought about by the starvation.

When pyruvate is added to slices of normal liver there is a great rise in the respiratory quotient, a large increase in oxygen consumption, and a large " negative glycolysis ", *i. e.* disappearance of acid. If pyruvic acid is oxidized according to the equation :

$$2\ C_3H_4O_3 + 5\ O_2 = 6\ CO_2 + 4\ H_2O,$$

the R.Q. of the process will be 1·2. If, in addition to pyruvic acid being burned, some is reduced to lactic acid, or converted into carbohydrate, which will also involve reduction ($2C_3H_4O_3 + 4H = C_6H_{12}O_6$), this process may still further raise the R.Q., because the reduction of the pyruvic acid will involve the oxidation of some other substance, and this may, in turn, have the effect of lowering the requirements of the whole tissue for atmospheric oxygen, without diminishing the amount of CO_2 produced. We may, therefore, tentatively interpret the respiratory quotient in the presence of pyruvate as indicating—

(1) That the respiratory metabolism of the tissue is probably concentrated on pyruvate.

(2) That some of the pyruvate is burned.

(3) That some of the pyruvate is converted into lactic acid or into carbohydrate.

Since 1 μl. of oxygen is required to burn $\frac{88 \times 2}{5 \times 22{,}400} = 0{\cdot}00157$ mg. of pyruvic acid, the total oxygen consumption (2229 μl. ; Table VIII) represents the consumption of 3·505 mg. of pyruvic acid.

Since the disappearance of one molecule of pyruvic acid will liberate one equivalent of base, which will take up one molecule of CO_2, a " negative glycolysis " of 1·0 μl. of CO_2 will correspond to the disappearance of $\frac{88}{22{,}400} = 0{\cdot}00393$ mg. of pyruvic acid, and the total " negative glycolysis " of −1379 μl. (Table VIII) will correspond to the disappearance of 5·417 mg. of pyruvic acid. (This argument of course, is subject to the reservations mentioned above, in discussing the general significance of the " glycolysis " figures.)

Now if 5·417 mg. disappear but only 3·505 mg. have been burned, it is possible that the difference, 1·91 mg., represents pyruvic acid synthesized to carbohydrate. If we compare this figure with the average figure given in Table I (where the experimental period was 2 hours), we find that the observed extra synthesis in 1 hour is $\frac{3{\cdot}52}{2} = 1{\cdot}76$ mg. The quantities are, therefore, of the right order.

We can also argue in the reverse direction. The formation of 1·76 mg. of carbohydrate from pyruvic acid will involve the participation of 2 atoms of hydrogen for every molecule of pyruvic acid, thus bringing about an amount

of oxidation of some other substances or substances (for instance, other molecules of pyruvic acid) (Krebs & Johnson, 1937) equivalent to 1 atom of oxygen per molecule of pyruvic acid reduced, and "sparing" the absorption of an equivalent amount of atmospheric oxygen, that is, $\frac{22{,}400 \times 1{\cdot}76}{88 \times 2}$, or 213 μl. The amount of CO_2 actually produced and oxygen actually consumed was 2934 μl. and 2229 μl. respectively. If to the oyxgen we add a further 213 μl., we obtain, as the R.Q., $\frac{2934}{2442} = 1{\cdot}20$. *But this is the R.Q. which we should obtain from the complete combustion of pyruvic acid.* We feel justified, therefore, in concluding tentatively that, of the pyruvic acid which is metabolized by slices of normal liver, some is completely burned, and the remainder is converted into carbohydrate. But it will be noted that this argument depends on the assumption that any carbohydrate synthesis other than that due to pyruvic acid entails neither a nett change in "glycolysis", nor the absorption of O_2, nor the production of CO_2.

In toxæmia, the oxygen consumption, the R.Q. and the "negative glycolysis" are all progressively decreased. It may be argued, therefore, that the machinery for oxidizing pyruvic acid as well as that for converting it into carbohydrate is somewhat damaged after 24 hours' toxæmia, and is still more severely damaged after 48 hours. The figure for carbohydrate synthesis from pyruvate after 48 hours' toxæmia is (Table II) 0·385 mg. per hour. From the "negative glycolysis" we may calculate that 1·14 mg. of pyruvic acid have disappeared. This would leave only 0·76 to be oxidized.

If we correct the R.Q. for carbohydrate formation, as we did above in the case of normal animals, we arrive at the figure $\frac{1290}{1601}$, or 0·806. This implies that substances other than pyruvic acid are now being oxidized. (Note, however, that oxygen consumption is still above the figure, 1408 μl., for toxæmic liver without added substrate.)

If we examine the figures in Table VIII which refer to experiments with lactate, we find them much more difficult to interpret. In the first place, although the R.Q. of the combustion of lactate is 1·0, and although the increases in oxygen consumption and carbohydrate synthesis with added lactate are nearly as large as that with added pyruvate, the rise in R.Q. is relatively small. This suggests that the metabolism of the tissue is not concentrated on lactate, as it appears to be on pyruvate when the latter substance is added. From Tables I, II, III and VIII together we can glean the following information :

TABLE IX.

	Lactic acid, disappearing, mg./g./hr.		Carbohydrate synthesized, mg./g./hour. (From Tables I and II.)
	By direct estimation (Table III).	Manometrically (Table VIII).	
Normal	1·56	2·23*	1·18
48 hours' toxæmia	0·16	1·07*	0·32

* 1 μl. CO_2 is equivalent to $\frac{90}{22{,}400} = 0{\cdot}004$ mg. of lactic acid.

1. In both normal and toxæmic animals, the disappearance of lactic acid deduced from the manometric measurements ("negative glycolysis") is considerably greater than that measured directly by chemical estimation. This illustrates the difficulty of interpreting manometric measurements which stand by themselves.

2. The observed synthesis of carbohydrate is, in normal animals, less than the disappearance of lactic acid, measured by either method. If we subtract the figure for the observed synthesis of carbohydrate from that for the disappearance of lactic acid measured by chemical estimation, we obtain 1·56 — 1·18 = 0·38, which might be supposed to represent lactic acid oxidized.

If we calculate the amount of O_2 absorbed and CO_2 evolved during the oxidation of this amount of lactic acid ($C_3H_6O_3 + 3\ O_2 = 3\ CO_2 + 3\ H_2O$) we obtain the figure 283 μl. for each. (1 mg. of lactic acid required $\frac{3 \times 22{,}400}{90} =$ 746 μl. of O_2, and of course an equal amount of CO_2 will be evolved.)

The normal tissue, without added lactate, uses 1841 μl. of O_2 and gives out 1086 μl. of CO_2 per g. per hour. If we add 283 μl. to each of these quantities, we get an oxygen consumption of 2124 μl. per g. per hour, and an R.Q. of 0·64. The observed O_2 consumption and R.Q. with added lactate are 2230 μl. and 0·65 respectively. The correspondence between the results is thus not at all bad. But, of course, the discrepancy would be considerable if we were to calculate the amount of lactic acid oxidized from the *manometric* figures instead of from those obtained by direct chemical estimation.

In the case of toxæmic animals, on the other hand, the figure for the observed synthesis of carbohydrate is *greater* than that for the disappearance of lactic acid observed directly. Without further data, no reason can be given for this. We may suspect that, in these circumstances, carbohydrate may be being manufactured from another substance which does not function so readily as a source of carbohydrate in the normal animal.

It should be emphasized again that the conclusions which have been drawn in this discussion must be of a tentative nature, until further work has given us a more detailed knowledge of the processes which occur. As they stand, however, they provide evidence that the normal liver, at least, does actually convert lactic and pyruvic acids to carbohydrate, and they also show that toxæmia affects the respiratory processes of the tissue, as well as its power of gluconeogenesis. Moreover, toxæmia appears specifically to affect the metabolism of these three carbon compounds. It is therefore very unlikely that we are dealing merely with the results of general cell destruction, which are more or less severe according to whether a greater or smaller number of cells are affected.

The results reported in this paper show that, in diphtheritic toxæmia, the metabolism of the liver is definitely altered, whether the alteration be due to damage to the chemical organization of the liver-cells themselves, or to the removal or modification of the hormonal influence to which they are normally subjected, or to both causes. The results are, so far, of a preliminary nature, and many lines of investigation which may be expected to prove fruitful still lie open. It is, moreover, clear that the methods which are now being developed in the field of biochemistry may be profitably directed to the

investigation of the behaviour of other organs, and to the effect on them of toxic agents other than those of the diphtheria bacillus.

SUMMARY.

1. The power of the isolated liver tissue of normal rabbits, and of rabbits in various stages of diphtheritic toxæmia, to form carbohydrate from various substances has been investigated and compared. The power to form extra carbohydrate in the presence of lactic acid, pyruvic acid and alanine is progressively reduced in toxæmia. Synthesis in the presence of glycerol and glycerophosphate and glyceric aldehyde are also affected, but to a much less degree.

The normal liver also forms some extra carbohydrate in the presence of butyrate, succinate and glycerate, but not in the presence of acetate.

2. The inhibition of carbohydrate formation by insulin *in vitro* is abolished by toxæmia.

3. It can be shown that slices of toxæmic liver cause less disappearance of lactic acid *in vitro* than do those of normal liver.

4. Corkill's finding that no glycogen is deposited in the liver of the whole animal after injections of lactate in early diphtheritic toxæmia is confirmed. Deposition of glycogen in the livers of such animals after injections of glucose seems however, to be diminished, though not abolished.

5. A small glycogen synthesis *in vitro* has been demonstrated. This is abolished by toxæmia, and by the addition of adrenaline *in vitro*.

6. Manometric experiments show that the addition of pyruvic acid to normal liver slices increases the oxygen consumption and respiratory quotient. Acid disappears. It is tentatively concluded that in these circumstances the metabolism of the tissue is concentrated on pyruvic acid, which is partly burned and partly synthesized to carbohydrate. In toxæmia both these processes are diminished, and other substrates besides pyruvic acid are metabolized. On the other hand, lactic acid does not, in the normal liver, replace other substrates in the same way as does pyruvic acid. But in toxæmia, diminution both of lactic acid combustion and of synthesis of lactic acid to carbohydrate are reflected in the results of manometric experiments.

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REFERENCES.

Bach and Holmes.—(1937) *Biochem. J.*, **31**, 89.
Blaschko and Schlossmann.—(1936) *Nature*, **137**, 110.
Cori and Cori.—(1933) *J. biol. Chem.*, **100**, 323.
Cori and Shine.—(1936) *Ibid.*, **114**, xxi.
Corkill.—(1932) *J. Physiol.*, **75**, 381.
Idem and Ochoa.—(1934) *Ibid.*, **82**, 399.
Cramer.—(1926) *Brit. J. exp. Path.*, **7**, 95.
Dixon.—(1934) ' Manometric Methods ', Camb. Univ. Press.

EVANS AND ZWECKWER.—(1926) *Brit. J. exp. Path.*, **8**, 280.
FRIEDMANN, COTONIO AND SHAFFER.—(1927) *J. biol. Chem.*, **73**, 335.
GEMMILL AND HOLMES.—(1935) *Biochem. J.*, **29**, 338.
GOOD, CRAMER AND SOMOGYI.—(1933) *J. biol. Chem.*, **100**, 485.
HAGEDORN AND JENSEN.—(1923) *Biochem. Z.*, **135**, 46.
KREBS & JOHNSON.—(1937) *Biochem. J.*, **31**, 645.
LAWRENCE AND BUCKLEY.—(1927) *Brit. J. exp. Path.*, **8**, 58.
MINOT.—(1931) *J. Pharmacol.*, **43**, 295.
Idem, DODD AND SAUNDERS.—(1934) *J. clin. Invest.*, **13**, 917.
THADDEA.—(1935) *Klin. Wschr.*, **14**, 1275.
YANNET AND DARROW.—(1933*a* and *b*) *J. clin. Invest.*, **12**, 767, 779.
Idem AND GOLDFARB.—(1933) *Ibid.*, **12**, 787.

THE ARTIFICIAL OPSONIZATION OF BACTERIA.—PART II.

J. GORDON AND F. C. THOMPSON.

From the Department of Pathology and Bacteriology and the Procter International Research Laboratory, University of Leeds.

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IN a previous publication, Gordon and Thompson (1936), working with staphylococci, showed that various tanning agents were capable of opsonizing this organism. The tanning agents included gallotannic acid and many other commercial vegetable tannins, and also inorganic tanning materials such as the basic salts of iron, chromium and aluminium. This work confirmed many of the earlier observations of Reiner, Fischer and Kopp (1929), of Freund (1929), and of Neufeld and Etinger-Tulcyznska (1929). It was observed that the tanning materials as a rule caused agglutination of the organism, and that when agglutination did not take place there was no subsequent phagocytosis. In view of the obvious relationship between the phenomena of agglutination and phagocytosis, it was thought desirable to extend the work to a more easily agglutinable organism, *B. typhosus*.

In extending our search for artificial opsonins we have included dye-stuffs and various synthetic tanning agents that are now extensively used in the leather trade. Many of the latter are condensation products of sulphonic acids of cresol and of naphthalene with formaldehyde. These contain sulphonic groups, and their tanning action is intimately connected with such groups. In this respect they very much resemble acid dye-stuffs, and the synthetic tannins may be regarded as " colourless acid dyes " or the acid dyes might be regarded as coloured synthetic tannins.

The technique used was as follows :

The strain of *B. typhosus* used was " Maire de Courcelles ", a strain isolated by one of the mobile laboratories during the war and kept in culture in this laboratory for almost 20 years. Forty-eight-hour growths on agar were used and a suspension of the growth was made in sterile

distilled water to a strength of about 500 millions per c.c.; 1 c.c. portions of this suspension were pipetted into a series of tubes, centrifuged, and the supernatant fluid removed. After the addition of 1 c.c. of the solution of the reagent to be tested as an opsonin, the mixture was shaken up and allowed to stand on the bench for 3 hours. The degree of agglutination was then noted. The tubes were next centrifuged, the supernatant fluid pipetted off and the deposit of bacteria washed several times with distilled water. Finally the bacteria were diluted to the original volume with distilled water. The purpose of the washing is to remove any excess of the opsonizing agent which might have a deleterious influence on leucocytes.

To a few drops of this bacterial suspension was then added a drop of a washed leucocytic cream obtained by the method described in the previous paper (Gordon and Thompson, 1936). The tubes of leucocytes and bacteria were incubated for 3 hours at 37° C. and films were made and stained by Leishman's method. Control tubes of bacteria treated with saline alone were always set up.

In these experiments with *B. typhosus*, spontaneous phagocytosis was a comparatively rare phenomenon.

Experiments were carried out on a large number of salt solutions and the results showed that these could be classified as follows:

(a) *Salts showing both agglutination and opsonization.*

These salts all possess tanning properties and all have been used for the commercial production of leather. They are the double sulphates known as alums, and arranged in order of increasing activity are: potash alum (aluminium salt), chrome alum (chromium salt), iron alum (ferric salt). The concentrations employed were 0·1 p.c.

(b) *Salts showing agglutination but with little or no opsonic activity.*

These were salts of heavy metals, such as lead nitrate and copper sulphate. The concentrations used were 0·5, 0·2 and 0·1 p.c. Lead and copper salts have practically no tanning action on skin.

(c) *Salts which neither agglutinate nor opsonize.*

Barium chloride, calcium chloride, magnesium chloride, magnesium sulphate, ferrous ammonium sulphate, nickel sulphate. Zinc and cadmium sulphates occasionally gave a trace of agglutination. The concentrations employed were the same as those in (b) above. All these salts are devoid of tanning power, and in this connection it is interesting to note that ferrous salts do not tan, but ferric salts have great tanning powers.

(d) *Buffer solutions containing acid which caused agglutination but no opsonization.*

Various buffer solutions were prepared by the partial neutralization of a mixture of acetic and formic acids in equi-molecular proportions. The optimum pH for agglutination of *B. typhosus* was found to be 3·9. These buffer solutions have no tanning properties whatever and showed no opsonization. There is little doubt that the agglutination is brought about by the removal of the electrical charges on the bacteria, and the pH of maximum agglutination might be regarded as the isoelectric point of the organisms.

Experiments with solutions of vegetable tannins as described in Part I (Gordon and Thompson, 1936), varying in concentration from 0·5 to 0·1 p.c., were carried out on this strain of *B. typhosus*. The results obtained showed a certain amount of irregularity, the agglutination being at one time considerable and at other times only slight. It was noteworthy that the degree of opsonization varied with the degree of agglutination.

Despite the well-known tanning properties of the so-called synthetic tannins, we had little success either with agglutination or opsonization on *B. typhosus*. This may be due to the fact that the synthetic tannins are used in acid solution

in the processes of leather manufacture, and the pH for successful tanning is so low that such solutions are destructive to the bacteria. If the solutions of the synthetic tannins are used at higher pH values their tanning action is materially reduced.

A further set of experiments were carried out with solutions of dye-stuffs. Most of those employed were basic dyes such as methylene-blue, magenta, methyl violet and gentian violet. These were used as salts, generally chlorides. Two acid colours, methyl orange and orange II, were used and also congo red, which is a direct cotton dye but behaves like an acid dye, combining with the proteins by its sulphonic group. Neutral red, a basic dye, was also tried. The dyes were used in concentrations of 0·1, 0·05 and 0·02 p.c. in distilled water. 1 c.c. of these solutions was added to the bacterial deposit from 1 c.c. of bacterial suspension; the mixture was shaken and allowed to stand on the bench for 3 hours. The tubes were then centrifuged, the supernatant fluid pipetted off and the bacteria washed several times.

Using *B. typhosus* we obtained no opsonization with these dye-stuffs, but in view of the findings of Neufeld and Etinger-Tulcyznska (1929) that solutions of methylene-blue and neutral red would opsonize pneumococcus, the experiments were repeated on various strains of staphylococcus. With some strains, but not with all, the dyes acted as artificial opsonins, and in particular the dye-stuffs with basic characters were best.

The special case of congo red calls for comment. Certain strains of staphylococcus were opsonized. It has been demonstrated previously by Gordon (1930), and by Gordon and Thompson (1935), that congo red in concentrations just sufficient to inhibit serum complement did not prevent the opsonic action of the serum. The action of congo red as an opsonizing agent itself on the organism should now therefore be taken into account in interpreting the previous results.

Since basic dye-stuffs can opsonize some strains of staphylococcus, and also since normal serum acts as an opsonin, other proteins of a predominantly basic character were sought that might act as artificial opsonins. The most basic proteins known are the protamines, and over thirty years ago Kossel and Dakin (1904) found that various protamines yielded on hydrolysis about 87 p.c. of arginine. This means that in the protamine molecule, two out of every three amino-acids must be arginine residues built into the main chain of the protein by means of the usual —CO—NH— linkages. The strongly basic group in the guanidine end of the arginine molecules is presumably free and consequently salts of the protamines were employed. Two samples were available, "protamine sulphate" (Burroughs Wellcome) and "salmiridine sulphate" (B.D.H.). Solutions of these were employed at concentrations of 1·0, 0·5, 0·25 and 0·1 p.c. With *B. typhosus* there was neither agglutination nor opsonic action, but with some strains of staphylococcus there was both marked agglutination and also opsonization.

There is thus a parallelism between the action of basic dyes and protamine salts as opsonins. A positive reaction was obtained with staphylococcus and a negative one with *B. typhosus*. The active groups are obviously the basic groups, and the results obtained appear to suggest that there are more reacting acidic groups in staphylococcus than in *B. typhosus*, and these react with

the basic groups in the dyes or protamine salts. A further possible development of this work is to use basic dyes or protamine salts as "indicators" for ascertaining the nature of the reacting groups of the organisms.

The natural opsonic power of normal serum appears to be simulated by the protamines. Since opsonization has never been noted without preliminary agglutination, the conclusion may safely be drawn that agglutination is necessary before opsonization may occur. Numerous workers have noted the mutual precipitation of proteins. For instance Beth af Ugglas (1914) showed that the protamine, clupein, precipitates hæmoglobin and casein. Michaelis and Davidsohn (1912) showed that the condition for most complete mutual precipitation of two proteins was a pH value of the solution intermediate between the isoelectric points of the reacting proteins. This is really equivalent to the theory of neutralization of opposite charges.

The mechanism of the action of gallotannic acid and commercial tannins upon organisms has already been discussed at length (Gordon and Thompson, 1936). A similar action is brought about by acid dyes and synthetic tannins, and this may be represented schematically as follows :

The protein in its " Zwitterion " form first reacts with acid,

$$R\begin{matrix}COO^- \\ NH_3^+\end{matrix} + H^+A^- \rightarrow R\begin{matrix}COOH \\ NH_3^+\end{matrix} + A^-,$$

and the anion becomes an uncharged carboxyl group, but the cationic NH_3 group is still left with a positive charge.

The acid dyes or synthetic tannins in the form of sodium salts are ionized, and the second stage of the reaction results in the formation of a non-hydrolysable compound between the positively charged protein and the negatively charged sulphonic ion of the dye or of the synthetic tannin :

$$R\begin{matrix}COOH \\ NH_3^+\end{matrix} + D.SO_3^- Na^+ \rightarrow Na^+ A^- + R\begin{matrix}COOH \\ NH_3 .D.SO_3.\end{matrix}$$

It will be noticed that acid is necessary to convert the protein " Zwitterion " into a cation before the reaction can proceed. In dyeing, the acid is added to the dyebath after the wool or leather has been run for some time in a solution of the dyestuff. With synthetic tannins however there is always acid present in the materials as sold.

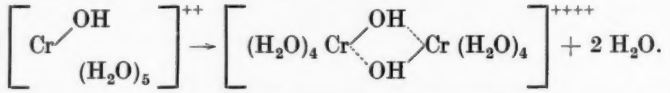
The action of the metallic salts of iron, chromium and aluminium is brought about in a different manner. All these salts undergo hydrolysis in water with the formation of free acid and a basic salt. For example the hydrolysis of chromium sulphate may be represented as follows :

$$\left[Cr(H_2O)_6\right]_2^{+++}\begin{matrix}SO_4^= \\ SO_4^= \\ SO_4^=\end{matrix} + H_2O \rightarrow H_2SO_4 + 2\left[Cr\begin{matrix}OH \\ (H_2O)_5\end{matrix}\right]^{++} + 2\,SO_4^=.$$

Normally an equilibrium would be set up in which the free acid would tend to react with the basic salt to regenerate the normal unhydrolysed chromium

sulphate. This equilibrium is prevented by the polymerization of the basic salt, which forms a binuclear (and eventually polynuclear) compound in which the oxygen atoms of the hydroxyl groups become co-ordinated with another metal atom. Such compounds have been termed " olated " (Stiasny, 1931), and are much more resistant to acid than un-olated basic salts.

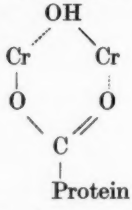
The process may be illustrated as follows :



The linkages marked by the dotted lines represent co-ordinated valencies and correspond to the " auxiliary valencies " of Werner.

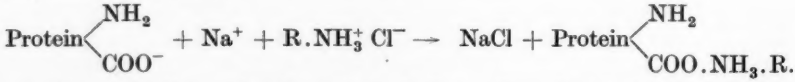
Olated compounds readily combine with ions such as sulphates, sulphites, acetates, formates and carboxylic ions generally. The ions of α-hydroxy carboxylic acids are a special case, but of these it need only be said here that their iron and chromium compounds are devoid of tanning action.

As proteins possess free carboxyl groups the process of tanning with iron, chromium and aluminium salts may be represented as—



in which the carboxyl anion of the protein has entered into combination with the olated metallic complex resulting in the formation of a non-hydrolysable compound.

The reaction with basic dyes is a combination of the coloured cation with the carboxyl anion of the protein, again with the formation of a non-hydrolysable compound :



The reaction with the protamine salt is similar. Here the protamine sulphate has a positively charged cation which reacts with the negatively charged anion of the protein of the organism.

The reactions described above will occur on the surfaces of the organisms, and so bring about the changes which allow of phagocytosis by the leucocytes. The reason why the leucocytes should engulf these changed organisms is still obscure.

SUMMARY.

The previous work on tanning agents as artificial opsonins has been extended to include experiments with *B. typhosus*. The most constant and regular in their action as opsonins have been the salts of iron and chromium.

It has been noted that agglutination and opsonization are intimately related, and that artificial opsonins always act as agglutinins, but that substances which agglutinate need not necessarily act as opsonins. The action of vegetable tannins as artificial opsonins has been found to be positive, but much more irregular with *B. typhosus* than with staphylococcus. Because dye-stuffs form non-hydrolysable compounds with proteins, their action as artificial opsonins has been investigated. While no effects could be demonstrated against *B. typhosus*, with some strains of staphylococcus there was undoubted opsonization. A further interesting result was obtained in that protamine sulphate agglutinated and opsonized staphylococcus.

We are pleased to express our indebtedness to our colleague, Mr. W. R. Atkin, for many valuable suggestions, and to the Medical Research Council for a grant towards the expenses of this work.

REFERENCES.

FREUND, J.—(1929) *Proc. Soc. exp. Biol.*, N.Y., **26**, 876.
GORDON, J.—(1930) *J. Immunol.*, **19**, 303.
Idem AND THOMPSON, F. C.—(1935) *Brit. J. exp. Path.*, **16**, 101 ; (1936) *ibid.*, **17**, 159.
KOSSEL, A., AND DAKIN, H. D.—(1904) *Hoppe Seyl. Z.*, **41**, 407.
MICHAELIS, L., AND DAVIDSOHN.—(1912) *Biochem. Z.*, **47**, 59.
NEUFELD, F., AND ETINGER, TULCYZNSKA, R.—(1929) *Zbl. Bakt.*, I Abt., Orig., **114**, 252.
REINER, L., AND FISCHER, O.—(1929) *Z. ImmunForsch.*, **61**, 317.
REINER, L., AND KOPP, H.—(1929) *Ibid.*, **61**, 397.
STIASNY, E.—(1931) ' Gerbereichemie—Chromgerbung ', Steinkopf (Dresden).
AF UGGLAS, B.—(1914) *Biochem. Z.*, **61**, 469.

SERICITE AND SILICA: EXPERIMENTAL DUST-LESIONS IN RABBITS.

S. LYLE CUMMINS.

From the Tuberculosis Department, Medical School, Cardiff.

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THE following experiments, planned on lines similar to those of Fallon and Banting (1935), were undertaken as a study of the action of sericite and silica respectively in animal tissues, and with the intention of ascertaining the effects, if any, of dead tubercle bacilli in addition to these minerals. I have to thank Dr. W. R. Jones, of the Royal School of Mines, whose investigations first called serious attention to the possibility of danger from the inhalation of sericite dust (1933), for supplies of sericite and quartz dusts of great purity and small particle size. The dust samples used were sent in two lots, a first, consisting

of rather coarser particles, many exceeding 10μ ; and a second, more finely ground, in which all the particles had been reduced to 10μ and under, many being only just visible with crossed prisms.

The experiments fall into two parts, the subcutaneous injection of the rather coarser dusts suspended in water into the ears of rabbits, and the intratracheal inoculation of similar suspensions of the finer dust samples, after the manner of Kettle, the animals being anæsthetized with ethyl urethane.

SUBCUTANEOUS INJECTIONS.

Suspensions of 1 g. each of sericite and quartz respectively in 5 c.c. of sterile distilled water were prepared and autoclaved. Volumes of approximately 0·1 c.c. were then injected into the ears of rabbits as follows, in some cases with and in others without the addition of a small volume of a grey emulsion of dead " human " tubercle bacilli :

Rabbit No. 289 : Right ear, sericite.
Left ear, quartz.
Rabbit No. 290 : Right ear, sericite.
Left ear, quartz.
Rabbit No. 295 : Right ear, sericite with dead tubercle bacilli.
Left ear, quartz with dead tubercle bacilli.
Rabbit No. 296 : Right ear, sericite with dead tubercle bacilli.
Left ear, quartz with dead tubercle bacilli.

It cannot be claimed that the volumes injected were exact. It was not easy to maintain the emulsion homogeneous in the syringe, and the subcutaneous tissue of the different animals was not equally resistant ; but it may be said that each animal received approximately 20 mg. of the dust employed with a margin of error that may perhaps be ignored in view of the considerable degree of uniformity of the resulting lesions. Rabbit 289, in which the first inoculation appeared to fall short of the intended volume, was at once given a second dose close by. In the event, there was not much difference in the size of the two swellings. The lesions produced consisted of smooth globular elevations, at first semi-fluctuating, later tending to become more solid. At the end of 4 weeks all the swellings were measured and the results recorded as follows :

Rabbit No. 289 : Right 14 × 10 × 3 mm., 10 × 9 × 3 mm. ; left 11 × 8 × 2·5 mm.
Rabbit No. 290 : Right 11 × 11 × 3·5 mm. ; left 7 × 5 × 1·2 mm.
Rabbit No. 295 : Right 14 × 11 × 5 mm. ; left 10 × 11 × 3 mm.
Rabbit No. 296 : Right 14 × 10 × 3 mm. ; left 12 × 8 × 3 mm.

These dimensions did not alter appreciably in subsequent months, except in two instances where the swelling broke down and discharged its contents.

It will be seen that, on the whole, the swellings produced by sericite were rather larger than those produced by quartz, but the difference was not greater than might be accounted for by slight errors in the volumes introduced. It is to be noted as important, however, that the swellings produced by pure

sericite and pure quartz, as well as those produced by quartz and dead tubercle bacilli, remained unbroken and unchanged up to the end of the sixth month; but the swellings in the right ears of Rabbits Nos. 295 and 296, resulting from sericite along with dead tubercle bacilli, both broke down some 8 weeks after injection and discharged their contents, then healed rapidly, leaving, after 6 months, only a flat and wrinkled area instead of a globular swelling. All 4 rabbits were killed at the end of 6 months, the ears removed and sections of the swellings prepared and examined.

Apart from the fact that the "quartz plus tubercle" lesions showed areas of caseation and numerous well-preserved tubercle bacilli, the cellular reactions in all the swellings were very similar. The lesions were strictly localized between the overlying epidermis and the ear cartilages, and consisted of large giant-cells and mononuclears packed with dust-particles and separated by an inter-cellular colloidal or gelatinous matrix, in which, in the quartz lesions, there was commencing fibrosis, as revealed by Van Gieson's and Mallory's stains, whereas, in the sericite lesions, fibrosis was slight or absent. The greatest difference lay in the appearances as seen by crossed prisms, the elongated fibrous crystals of sericite presenting a marked contrast to the rounder, more plate-like and less brilliantly birefringent particles of quartz.

The giant-cells were more numerous in the quartz lesions, the large mononucleated cells in the sericite. In both, the cells, while well preserved as to outline and nuclei, gave, in their staining reactions, the impression of being dead and, in the words of Mavrogordato (1922), "mummified", an impression which was given also by the cells in the lung lesions about to be described.

INTRA-TRACHEAL INOCULATION.

Whereas Fallon and Banting had introduced dusts into the bronchi by means of a fine catheter passed down the trachea, it was decided in the present series to employ Kettle's method of exposure of the trachea in the anæsthetized rabbit and direct intratracheal inoculation.

For these experiments specially fine dust samples of under 10μ were obtained from W. R. Jones and suspended as before in a strength of 1 g. to 5 c.c., the fluids being autoclaved for sterility and homogenized in a "shaker" just before injection. The suspending fluid chosen was "borate buffer" solution, as this was thought less likely to irritate.

The animals were anæsthetized with ethyl urethane, 25 p.c. W/V, 62·5 g. to 250 c.c., kindly prepared by Dr. Hethcote, a dose of 5 c.c. per kg. of body-weight being given subcutaneously in the mid-dorsal region.

This amount kept the rabbits in a state of deep somnolence for 5 or 6 hours. The operations were done 2 hours after ethyl urethane injection, and the animals were laid on their right sides so as to facilitate aspiration of the inoculum into the right lung as far as possible. Rabbits Nos. 291 and 292 received sericite, Rabbits Nos. 293 and 294 quartz. Five weeks after the intra-tracheal treatment with dust, Rabbits Nos. 292 and 294 were each given intravenously 0·5 c.c. of a suspension of "human" tubercle bacilli killed by heating to 100° C. for half an hour, strength 1 mg. to 1 c.c. It was hoped that, by this time, dust lesions would be in process of formation, and that the dead

tubercle bacilli, carried to the lungs in the venous circulation, might produce some visible effect on or around the sericite and the quartz lesions respectively, as compared with the lesions in the rabbits receiving the pure dusts only.

All four animals were killed 6 months after treatment; the naked-eye appearances at post-mortem were as follows:

Rabbit No. 291: Very little if any abnormality visible in lungs. A "collar" of white induration around trachea.

Rabbit No. 292: In right lung, upper and middle lobes, areas of consolidation with white infarct-like spots.

Rabbit No. 293: Right upper, middle and lower lobes, consolidated white areas suggesting broncho-pneumonia, definitely more marked than those in Rabbit No. 292.

Rabbit No. 294: Right upper, middle and lower lobes show areas of consolidation, as do parts of left lung in less degree. The lesions are confluent in the right upper and mottle the section along the bronchial tree in the middle lobe. This animal is the most severely affected of the four.

Blocks for section were made from appropriate parts of all lobes of both lungs and through the superior mediastinal area, as well as such other tissues and organs as seemed advisable. Apart from the "collar" of peri-tracheal induration in No. 291, which proved to consist of an extensive sericite swelling like those described in the ears in the first experiment, only the lungs were found abnormal and no description of other organs is given. It is to be regretted that the injection in Rabbit No. 291 miscarried, as this accident rendered impossible the hoped-for comparison with No. 292. As no abnormal appearance suggesting tuberculosis was noted, however, in or around the lung lesions in Rabbit No. 292, it may be assumed that the effect of the injection of dead bacilli was negative or transitory around the sericite lesions, which may therefore be regarded as comparable with those of Rabbit No. 293 receiving quartz alone.

Rabbit No. 294, receiving quartz intra-tracheally, followed 5 weeks later by tubercle emulsion intra-venously, is in a category by itself. Here the lung tissue around the dust-lesions showed peri-focal inflammatory reaction and cellular infiltration which, although not suggesting tuberculous tissue, was still very much what occurs around tubercles after a tuberculin inoculation. It would be hard to deny that the post-coniotic tubercle injection was specifically active around the quartz dust-nodules in this animal.

In Rabbit No. 294, also, the section through the upper part of the mediastinum contained a satisfactory portion of a lymphatic gland in which definite silicotic deposition was found to have occurred. Unfortunately the mediastinal sections from the other rabbits were not satisfactory, and it was impossible from them to establish or to exclude the passage of dust-laden phagocytes to the tracheo-bronchial glands.

The histological characters of the lung lesions produced by sericite and quartz dust respectively are illustrated in Figs. 1 and 2, in which microphotographs of typical nodules from Rabbits Nos. 292 and 293 are reproduced, as seen (*a*) in sections stained with logwood and eosin and (*b*) by means of "crossed prisms". The close resemblance between the cellular reaction excited by the two dusts is evident at a glance. Without the conclusive evidence afforded by the characters of the crystals visible in the dust-cells, it would be difficult or

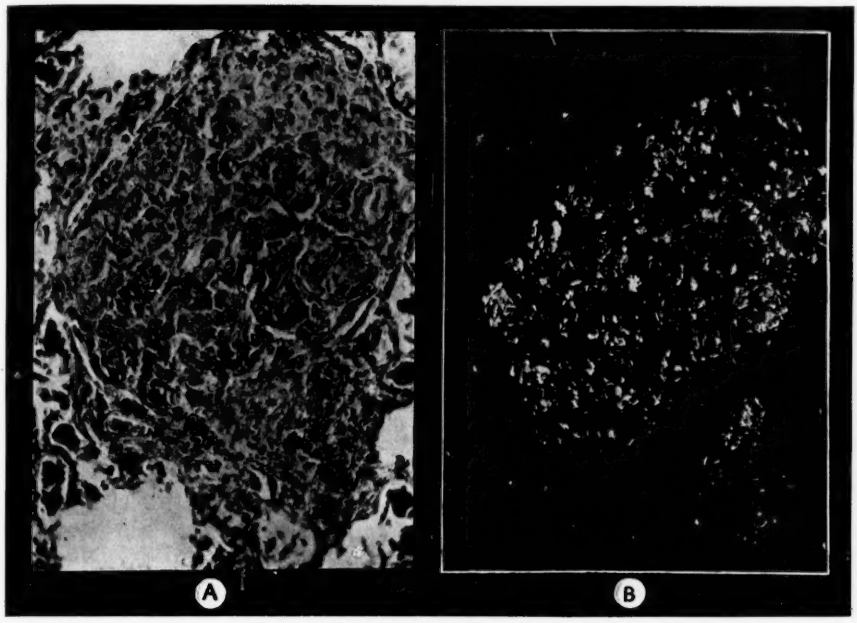

FIG. 1. × 100.—A. Sericite nodule from Rabbit No. 292. B. The same nodule seen through crossed prisms.

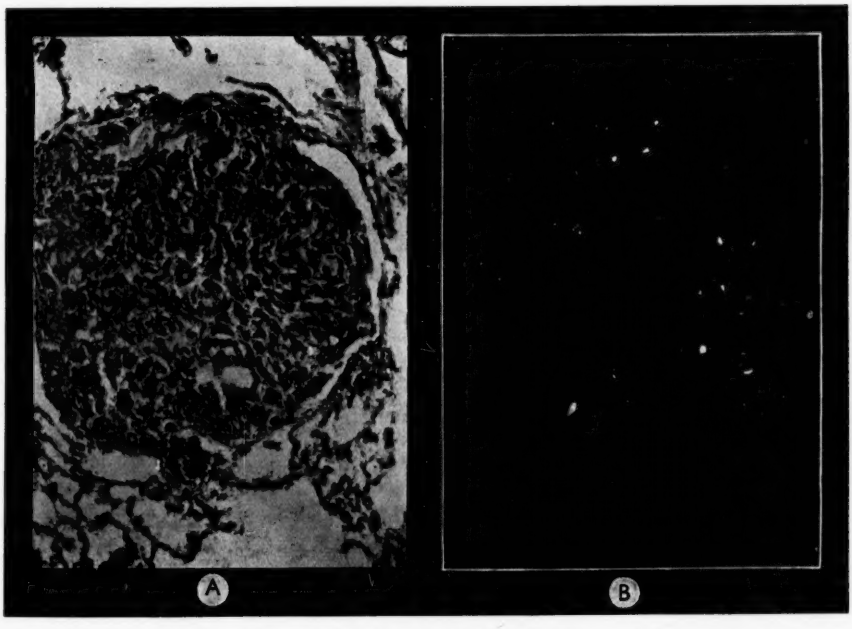

FIG. 2. × 100.—A. Quartz dust nodule from Rabbit No. 293. B. The same nodule seen through crossed prisms.

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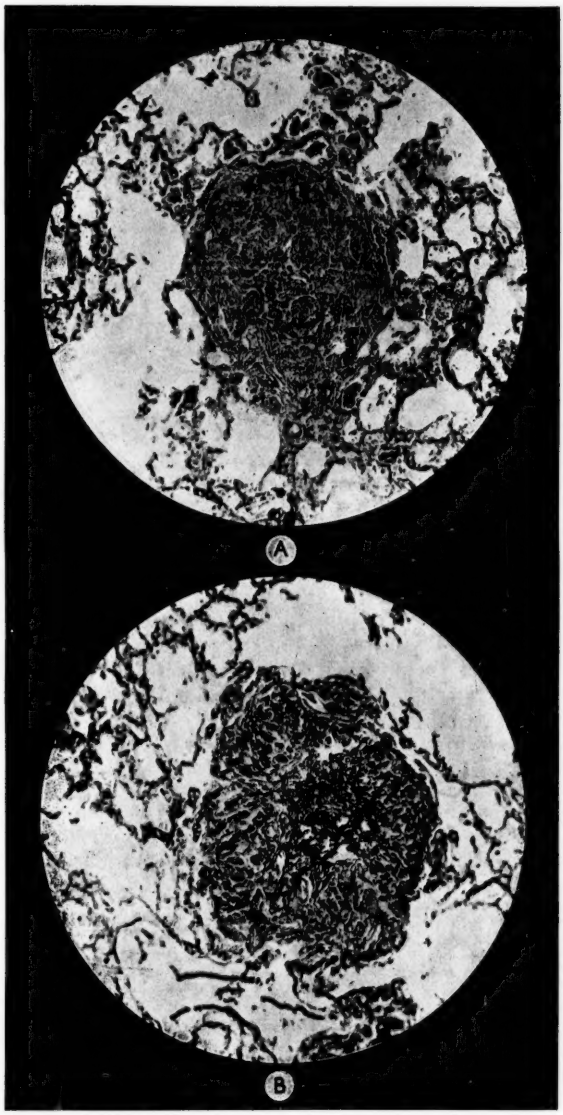

FIG. 3. × 60.—A, Sericite nodule, and B, quartz dust nodule stained with Mallory's aniline blue stain. Note the central fibrosis in B and the absence of any similar fibrosis in A.

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impossible to distinguish the one from the other. There is, perhaps, a little more perifocal inflammation about the sericite nodule illustrated than around the quartz nodule, but this was not always to be seen. A more real difference lies in the tendency to some degree of fusion of the outlines of the dust-cells in the centre of the quartz lesion, suggesting early necrotic change ; and a comparison of the photograph with crossed prisms indicates that while the micaceous particles are evenly distributed throughout the lesion both in number and in size, the quartz particles are more numerous and larger at the periphery, smaller and fewer towards the centre, as noted by Belt (1929) in human silicotic lesions. This suggests that some degree of surface solution has taken place in the central part, just where the early necrotic changes are to be seen. These peculiarities of the silicotic lesion in Fig. 2 were not confined to a few nodules but were common to the majority. Their significance is increased by a study of these nodules by means of special fibrous tissue stains. In Fig. 3 a sericite nodule from Rabbit No. 292 and a quartz nodule from Rabbit No. 293, both stained with Mallory's aniline blue stain, are presented for comparison. It will be seen that the quartz nodule is permeated, at its central part, by a fine reticulum of fibres, whereas there is no such fibrotic change in the sericite nodule.

The writer has seen no previous reference to this central fibrosis in experimental silicosis, although many have described peripheral whorls such as are accepted as characteristic of human silicosis. It is, perhaps, significant that almost all the successful attempts to reproduce the typical silicotic nodule of man in laboratory animals, such as those of Gardner (1932), have depended upon the inhalation of dust over a long period. The intra-tracheal method differs from the natural inhalation of dust in being a single event instead of a long-drawn-out exposure to successive dustings. An inhalation lesion is, doubtless, constantly reinforced by freshly arriving dust-cells. In the words of Belt, " as it heals dust-cells will lay down more silica around its periphery and a progressive lesion ensue ". Perhaps central fibrosis is to be expected after intra-tracheal dusting. Kettle, indeed, figures a whorled nodule after simultaneous intra-tracheal injection of kaolin and dead tubercle bacilli ; but in this case the peripheral fibrosis strongly suggests a chronic tuberculous reaction.

The question of exactly how the dust-nodules above figured are produced is of some interest in this connection. Their localization was often noted as being in the position of the peribronchial and perivascular lymphatic spaces, and the impression was gained that they resulted from the accumulation of dust-laden alveolar phagocytes in these situations. If this is so, then the central cells would have been the first to become impacted in the lymph-channels, and would have been longest in position and therefore most likely to show fibrotic changes. In a guinea-pig similarly treated and dying of intercurrent disease on the fifth day after intra-tracheal dust-injection, the particles were found widely and evenly scattered throughout the right lung and not gathered into nodules—a fact which supports the " impaction of dust-cells " theory. On the other hand, giant-cells had penetrated deeply and engulfed the dust-particles in the ear lesions, so it may be that the lung nodules also resulted from phagocytic penetration of intra-bronchiolar or alveolar deposits.

The problem could only be settled by the killing and examination, at short intervals, of a succession of intratracheally treated rabbits.

DISCUSSION.

The lesions produced with sericite and quartz dust respectively in the above experiments correspond closely to those described by Fallon and Banting (1935), Lemon and Higgins (1933), Policard (1934) and, for the intraperitoneal route, by Millar and Sayers (1937). These and other observations make it clear that quartz dust is a more active agent than sericite in producing fibrosis. The question remains, however, whether sericite is to be regarded merely as " inert ", or as possessing dangerous properties of its own. Fallon and Banting regard the reaction to sericite as " comparable to that produced by innocuous foreign substances ". Millar and Sayers classify it, with anthracite, bituminous coal-dust and many other minerals as " inert ", nor do Lemon and Higgins regard it as active. Policard, on the other hand, says of his experiments that the inhalations of a micaceous dust regarded as sericite " were not harmless ".

The epidemiological findings of W. R. Jones (1933) are so suggestive that it seems premature to classify sericite as merely inert because certain animal experiments, limited in point of duration, and not always strictly in accord with the phenomena of dust inhalation in industry, show it to be less active than quartz in the production of fibrotic nodules. The fact is that it does produce nodules, almost exactly like those resulting from silica, save in respect of the more rapid production of fibrosis by the latter.

Mavrogordato (1922) has expressed the belief that " all dusts which invade the lung provoke the production of cells that may turn into white fibres and increase the amount of fibrous tissue ". This belief is supported by recent cell-culture experiments of J. K. Moen (1935), which showed the development of pure cultures of fibroblasts from single mononuclear cells. Mavrogordato adds : " It is the dust which cannot escape from the lung-tissues which is the source of danger." My findings suggest that sericite cannot escape more readily from the lung-tissues than quartz dust ; indeed the locking up of sericite in the lungs of man has been stressed by Jones and is easily demonstrated. It would appear that the simultaneous introduction of dead tubercle bacilli and sericite excited greater destruction of tissue than a similar inoculation of quartz and tubercle bacilli, though it should be added that intravenous inoculation of dead tubercle bacilli following intra-tracheal dusting led to no augmentation of sericite lesions, but to an inflammatory reaction around silica nodules. These findings may not be contradictory but merely complementary. Sericite and tubercle bacilli must often reach the lungs of industrial workers simultaneously and lie in close juxtaposition in the tissues. Nor does sericite ever get inhaled as a pure dust but always mixed with other minerals, some perhaps capable of augmenting and others of damping its activity. Further experiments are called for to settle the position of sericite in relation to pneumonoconiosis, and these should be planned to show whether sericite can so block up lymph-spaces and lymphatics as to imprison other dusts in the lungs, and whether its action augments or is augmented by that of tubercle bacilli when these are also present.

SUMMARY.

As already shown by Fallon and Banting and by others, sericite, like silica, when introduced into the lungs or under the skin, leads to nodules composed of collections of large mononucleated phagocytes and giant-cells, but it falls far behind silica as an agent for the production of fibrosis.

The simultaneous introduction of sericite and dead tubercle bacilli subcutaneously led, in the two rabbits so treated, to extensive necrosis and sinus formation. Its action, in this respect, was more destructive than that following the inoculation of silica dust and dead tubercle bacilli.

On the other hand, the late addition of dead tubercle bacilli intravenously led to no accentuation of sericite lung-lesions, but had an irritative effect around lung-lesions produced by silica.

Caution is advised in classifying sericite as "inert" without further experimental investigations.

REFERENCES.

BELT, T. H.—(1929) *Canad. publ. Hlth. J.*, **20**, 494.
FALLON, J., AND BANTING, F. G.—(1935) *Canad. med. Ass. J.*, **33**, 407.
GARDNER, LEROY U.—(1932) *J. industr. Hyg.*, **14**, 18.
JONES, W. R.—(1933) *J. Hyg.*, Camb., **33**, 307.
KETTLE, J. H.—(1934) *J. Path. Bact.*, **38**, 201.
LEMON AND HIGGENS.—(1933) *Amer. Rev. Tuberc.*, **28**, 470.
MAVROGORDATO, A.—(1922) *Publ. S. Afr. Inst. med. Res.*, **15**, 122.
MILLAR, J. W., AND SAYERS, R. R.—(1936) *Publ. Hlth. Rep. Wash.*, **51**, No. 47.
MOEN, J. K.—(1935) *J. exp. Med.*, **61**, 247.

METABOLISM AND ACTION OF DIIODOTYROSINE AND *n*-BENZOYLDIIODOTYROSINE.

I. SNAPPER AND A. GRUNBAUM.

From the Laboratory of General Pathology and the Clinic of Internal Medicine of the Municipal University of Amsterdam.

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To explain the favourable influence of both tincture of iodine and of diiodotyrosine on exophthalmic goitre the following possibilities have to be considered: (*a*) The iodine of the tincture is first synthesized to diiodotyrosine; (*b*) the diiodotyrosine is first broken down to inorganic iodine before a favourable action upon hyperthyroidism is possible. In this respect the fact that all substances, containing the group (as for example diiodotyrosine

and thyroxine) are immunochemically closely connected, might be important (Snapper and Grunbaum, 1936). Is this group of diiodotyrosine perhaps the specific part of the molecule which is responsible for the therapeutic action ?

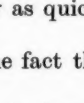
We observed that excretion of inorganic iodine starts a short time (about 50 minutes) after administration of diiodotyrosine. After oral or subcutaneous administration of 400 mg. of diiodotyrosine, inorganic iodine can be demonstrated in the saliva ; oral administration of 250 mg. of potassium iodide causes already positive iodine reactions in the saliva after 20 minutes.

The method for demonstration of inorganic iodine in the saliva (Heath and Fullerton, 1935) is as follows : The patient must chew a piece of cottonwool in order to collect the saliva. Two drops of a 10 p.c. $FeCl_3$ solution are added to 1 c.c. of saliva, together with 1 c.c. of a starch solution. The result is read after 30 minutes ; presence of iodine gives a blue colour.

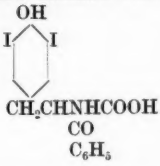
The excretion of inorganic iodine in the saliva after administration of diiodotyrosine lasts for about 24 hours, just as after administration of potassium iodide (see Table I). It must be remembered that unchanged diiodotyrosine can never be demonstrated in the saliva ; here the colour reaction of Kendall remains always negative even after subcutaneous injection of diiodotyrosine.

TABLE I.—*Inorganic I in Saliva.*

		250 mg. KI orally (200 mg. I).	400 mg. diiodotyrosine orally (240 mg. I).	150 mg. diiodotyrosine subcutaneously.
X (F.), 18 years	After 15 min.	—	—	—
	,, 25 ,,	+	—	—
	,, 50 ,,	+	+	—
	,, 3 hr.	+	+	slightly +
	,, 5 ,,	+	+	+
	,, 20 ,,	+	+	+
	,, 24 ,,	—	—	—
Y (F.), 19 years	After 20 min.	+	—	—
	,, 30 ,,	+	—	—
	,, 50 ,,	+	+	—
	,, 90 ,,	+	+	+
	,, 3 hr.	+	+	+
	,, 9 ,,	+	+	+
	,, 20 ,,	+	+	slightly +
	,, 27 ,,	—	—	—
Z (F.), 19 years	After 15 min.	+	—	—
	,, 40 ,,	+	—	slightly +
	,, 60 ,,	+	+	+
	,, 2 hr.	+	+	+
	,, 8 ,,	+	+	+
	,, 24 ,,	+	+	+
	,, 30 ,,	+	—	—

The fact that after administration of diiodotyrosine excretion of inorganic iodine in the saliva starts nearly as quickly and lasts nearly as long as after administration of KI points to the fact that the  I-group of diiodotyrosine is nearly instantly broken down to inorganic iodine. It is easily possible to stabilize the immunochemically important I-group of diiodotyrosine against the metabolizing powers of the body. Diiodotyrosine has to be considered as an aromatic *a*-amino-acid, which like all *a*-amino-acids is readily broken down in the body. However, thirty years ago Magnus Levy (1907) already proved that after benzoylation of the NH_2-group of an amino-acid, the benzoylated *a*-amino-acid is excreted unaltered. The same evidently holds true for benzoylated diiodotyrosine.

We prepared *n*-benzoyldiiodotyrosine by using the method of Sörensen (1908): 3·6 g. of diiodotyrosine are dissolved in 2·1 c.c. of water and 6·25 c.c. 2*N* NaOH. Under ice-cooling and mechanical stirring 4·66 g. benzoylchloride and 38 c.c. 2*N* NaOH are added. Thus after benzoylation the mixture contains about *N*/2 NaOH. The clear fluid is now acidified with 5 *N* hydrochloric acid, the precipitate is filtered off and washed with water. After being dried in a vacuum exsiccator above KOH the substance is boiled 5 times with petrol ether in order to remove benzoic acid. By crystallization with ether and petrol ether pure *n*-benzoyldiiodotyrosine (melting-point 179°) is obtained.



This *n*-benzoyldiiodotyrosine is excreted unchanged in the urine as well after oral as after subcutaneous administration.

The following method is used for the recovery of *n*-benzoyldiiodotyrosine from the urine: Urine is concentrated *in vacuo* until nearly dry. After addition of alcohol (10 times the volume) the precipitate is filtered off. This filtrate is again concentrated *in vacuo* at low temperature. After addition of hydrochloric acid it is shaken with ether; the ether is removed by aeration with an electrical fan. The rest is dried in the vacuum exsiccator. The residue is dissolved and neutralized with soda and filtered. To the filtrate a saturated solution of strychnine nitrate is added till a maximal precipitate is formed. The precipitate must remain during 1 hour at room temperature, after which it is centrifuged and washed once with saturated strychnine nitrate solution.

The precipitate is then dried in the vacuum exsiccator above H_2SO_4, after which it is heated with 10 c.c. *N* NaOH on a water-bath for 1 hour. Now the strychnine is split off and (as the reaction of the mixture is alkaline) precipitated. After 1 hour the strychnine is filtered off, the filtrate is acidified with hydrochloric acid, by which after recrystallization pure *n*-benzoyldiiodotyrosine is obtained with a melting-point of 179°.

This process of recovery of *n*-benzoyldiiodotyrosine from the urine involves heavy losses. As about 60 p.c. of the substance given can be recovered from the urine (Table II), it is probable that nearly all the *n*-benzoyldiiodotyrosine is excreted unchanged. This is the more probable as after administration of *n*-benzoyldiiodotyrosine no inorganic iodine can be found in urine and saliva. Prolonged administration of *n*-benzoyldiiodotyrosine does not change the

amount of the substance excreted (Table III); even in the long run *n*-benzoyldiiodotyrosine is not attacked in metabolism. If *n*-benzoyldiiodotyrosine is given to a patient no inorganic iodine is formed; now the action of the

-group may appear.

TABLE II.—n-*Benzoyldiiodotyrosine.*

	Injected (mg.).	Percentage excreted. 1st 24 hours.	2nd 24 hours.	3rd 24 hours.	4th 24 hours.
Dog . . .	500	53	7	6	1
A (F.), duod. ulc.	300	75	3·2	2·6	..
B (M.), ventr. ulc.	150	57	13	5	1
C (M.), duod. ulc.	2 × 125	61·3	7·8	3·5	2·3
D (M.), cc. ventr.	250	60·8	..	..	..
E (M.), ventr. ulc.	250	62·5	4·6	2·4	..

TABLE III.—n-*Benzoyldiiodotyrosine.*

	Subcutaneously injected.	Percentage excreted. I.	II.	III.	IV.	V.	VI.
F (F.)	During 6 days each day 25 mg.	50	74	70	60	80	96
G (F.), M. Basedow	During 14 days each day 50 mg.	40 excreted on the 14th day.					
H (F.), nephrolithiasis	During 9 days each day 50 mg.	53 excreted on the 9th day.					

TABLE IV.—n-*Benzoyldiiodotyrosine.*

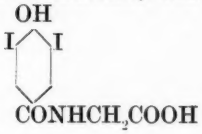
	Orally ingested.	Percentage excreted. I.	II.	III.
I (M.)	500 mg.	55·5	3·7	2·7
J (M.)	500 mg.	54·1	1·9	0·8
K (F.)	During 4 days each day 400 mg.	61 excreted on the 4th day.		

However, it appears that administration of *n*-benzoyldiiodotyrosine has no influence at all upon the basal metabolism of patients suffering from exophthalmic goitre (Table V). Administration of quantities varying from 25 to 500 mg. per day does not lower the basal metabolism nor influence the general condition of these patients, whereas administration of equivalent quantities of iodine or of diiodotyrosine causes slowing of the pulse, decrease of perspiration and improvement of the general condition.

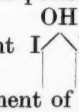
TABLE V.—*Administration of* n-*Benzoyldiiodotyrosine.*

	Daily.	Basal metabolism.		
L (F.) .	25 mg. subcutaneously, .	5.xi .	17.xi .	..
	26.x–17.xi.1935	+ 34%	+ 47%	
M (F.) .	2 × 50 mg. orally, .	20.v .	27.v .	5.vi
	21.v–9.vi. 1936	+ 51%	+ 54%	+59%
N (F.) .	50 mg. subcutaneously, .	11.i .	25.i .	..
	11.i–25.i.1936	+ 21.5%	+ 27%	..
O (F.) .	300 mg. orally, .	4.iv .	14.iv .	..
	4.iv–14.iv.1936	+ 36·5%	+ 84·3%	..
P (F.) .	2 × 250 mg. orally, .	13.xi .	18.xi .	1.xii
	14.xi–27.xi.1936	+ 100·6% ?	+ 49%	+ 67%
Q (M.) .	2 × 250 mg. orally, .	18.xi .	30.xi .	..
	19.xi–1.xii.1936	+ 69%	+ 72%	

Another iodine compound with closely related chemical structure, *i. e.* diiodooxyhippuric acid, is also not broken down in the metabolism ; administration of this substance does not cause excretion of inorganic iodine in the urine. This compound too has no influence upon basal metabolism or general condition of patients suffering from exophthalmic goitre.



SUMMARY.

Soon (about 50 minutes) after oral or subcutaneous administration of diiodotyrosine inorganic iodine can be demonstrated in the saliva. Thus in the human body diiodotyrosine must be broken down quickly to inorganic iodine. By benzoylation of the NH_2 group of the diiodotyrosine *n*-benzoyldiiodotyrosine is obtained, which appears unaltered in the urine. This new compound, however, has no influence upon the condition of the patients with exophthalmic goitre. Thus the immunochemically important  group of the diiodotyrosine cannot be the active group in the treatment of patients with hyperthyroidism. Probably the favourable influence of diiodotyrosine on patients with exophthalmic goitre is due to the fact that this substance is broken down quickly to inorganic iodine.

REFERENCES.

HEATH, C. W., AND FULLERTON, H. W.—(1935) *J. clin. Invest.*, **15**, 476.
MAGNUS LEVY.—(1907) *Biochem. Z.*, **6**, 541.
SNAPPER, I., AND GRUNBAUM, A.—(1936) *Ned. tijdschr. v. Geneesk.*, II, 2058.—(1936) *Brit. J. exp. Path.*, **17**, 361.
SÖRENSEN, S. P. L., AND ANDERSEN, A. C.—(1908) *Hoppe-Seyl. Z.*, **56**, 289.

THE ASSAY OF STAPHYLOCOCCUS ANTITOXIN BY THE HÆMOLYTIC METHOD.

W. AEG. TIMMERMAN.

From the Ryks Instituut voor de Volksgezondheid, Utrecht, Holland.

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STAPHYLOCOCCUS antitoxin is capable of neutralizing the hæmolytic, necrotic and lethal powers of staphylococcus toxin in a quantitative manner. It is therefore possible to make use of these properties of the toxin for the assay of the antitoxin. The results may be expressed in terms of the International Unit as established by the League of Nations. Titration by the hæmolytic method has advantages over the other two methods; it is accurate, quick, easy and cheap. It is frequently used in different laboratories (Parish, O'Meara and Clark, 1934; Glenny and Stevens, 1935; Hartley and Llewellyn Smith, 1936; Llewellyn Smith, 1936; McClean, 1937).

The question arises whether it is allowable to consider the resistance to hæmolysis of red cells equal in all rabbits. Apparently it has been generally assumed up till now that this is indeed the case. I cannot find any references in the current literature that the possibility of different behaviour of cells from different rabbits has been taken into consideration. In order to verify the correctness of this assumption, some experiments have been carried out, which will be discussed below. An attempt was made at the same time to obtain information as to whether the test dose of toxin, determined to a no-hæmolysis endpoint, varied more or less considerably than the test dose determined to a complete-hæmolysis endpoint.

It was originally planned that a test dose to a partial-hæmolysis endpoint should be used. This soon proved impracticable. Especially when titrations were carried out in tenths or hundredths of units of antitoxin there were often tubes in the series in which exactly the same degree of hæmolysis had taken place. This plan was therefore abandoned.

TECHNIQUE.

Red Blood Cells.—Well washed cells of 12 rabbits were used, all rabbits having less than 0·02 International Units of antitoxin in 1 c.c. of serum. One rabbit (No. 268) died during the experiments, its cells could therefore not be used for the determination of the test doses of toxin No. 19.

Toxins.—Three different filtered broth cultures, grown under carbon-dioxide have been used (Nos. 7, 17 and 19). The minimal hæmolytic doses were 0·0008, 0·0007 and 0·001 c.c. respectively. The filtrates did not contain β-toxin of Glenny and Stevens (1935). The question whether the a_1- and a_2-components (Morgan and Graydon, 1936) of a-toxin were both present has not been investigated, because this did not influence the results of these experiments.

Antitoxin.—For all experiments the International Standard was used, kindly provided by Dr. Claus Jensen from the Serum Institute of Copenhagen.

Mixtures and readings.—Mixtures were made up as follows: 0·5 c.c., containing the required amount of toxin was mixed with 0·5 c.c. of standard serum dilution, containing 1, 0·1 or 0·01 unit of antitoxin. This was left at room temperature for 30 minutes. Then 1 c.c. of a 2 p.c. suspension of cells was added, the mixture shaken and put in the incubator (37° C.) for one hour. After another hour on the laboratory bench readings were taken. All dilutions were made with 0·85 p.c. saline.

RESULTS.

Determination of Test Dose for 1, 0·1 *and* 0·01 *Unit* (*Tables I, II and III*).

TABLE I.—*Test Dose for* 1 *Unit of Antitoxin with Cells of* 12 *Rabbits.*

Rabbit.	Toxin No. 7.		Toxin No. 17.		Toxin No. 19.	
	No hæmolysis.	Complete hæmolysis.	No hæmolysis.	Complete hæmolysis.	No hæmolysis.	Complete hæmolysis.
268	0·11	0·12	0·12	0·14	..	..
295	0·11	0·12	0·12	0·14	0·11	0·14
296	0·11	0·16	0·12	0·16	0·12	0·14
297	0·11	0·14	0·10	0·12	0·12	0·14
312	0·12	0·14	0·12	0·14	0·12	0·16
313	0·12	0·14	0·12	0·14	0·12	0·16
314	0·12	0·14	0·10	0·14	0·12	0·14
315	0·12	0·14	0·10	0·14	0·12	0·14
316	0·12	0·14	0·10	0·12	0·12	0·14
317	0·12	0·14	0·10	0·14	0·12	0·14
318	0·12	0·14	0·10	0·12	0·12	0·14
319	0·12	0·14	0·10	0·12	0·12	0·14
Variations.	9%	33%	20%	33%	9%	14%

TABLE II.—*Test Dose for* 0·1 *Unit of Antitoxin with Cells of* 12 *Rabbits.*

Rabbit.	Toxin No. 7.		Toxin No. 17.		Toxin No. 19.	
	No hæmolysis.	Complete hæmolysis.	No hæmolysis.	Complete hæmolysis.	No hæmolysis.	Complete hæmolysis.
268	0·013	0·018	0·013	0·018	..	..
295	0·014	0·016	0·012	0·016	0·014	0·018
296	0·014	0·028	0·013	0·029	0·014	0·029
297	0·013	0·018	0·012	0·017	0·014	0·019
312	0·013	0·019	0·013	0·018	0·015	0·020
313	0·014	0·018	0·013	0·016	0·014	0·019
314	0·014	0·018	0·014	0·017	0·014	0·018
315	0·014	0·019	0·013	0·017	0·014	0·019
316	0·016	0·023	0·013	0·017	0·014	0·019
317	0·015	0·023	0·012	0·016	0·014	0·018
318	0·016	0·021	0·013	0·017	0·014	0·018
319	0·015	0·020	0·013	0·016	0·014	0·019
Variations.	23%	75%	17%	81%	7%	61%

TABLE III.—*Test Dose for* 0·01 *Unit of Antitoxin with Cells of* 12 *Rabbits.*

Rabbit.	Toxin No. 7.		Toxin No. 17.		Toxin No. 19.	
	No hæmolysis.	Complete hæmolysis.	No hæmolysis.	Complete hæmolysis.	No hæmolysis.	Complete hæmolysis.
268	0·0026	0·0042	0·0026	0·0046	..	..
295	0·0024	0·0034	0·0026	0·0044	0·0022	0·0036
296	0·0032	>0·0078	0·0028	>0·0078	0·0024	>0·0078
297	0·0024	0·0036	0·0024	0·0044	0·0022	0·0040
312	0·0028	0·0042	0·0024	0·0044	0·0022	0·0038
313	0·0028	0·0042	0·0024	0·0040	0·0022	0·0038
314	0·0028	0·0044	0·0022	0·0036	0·0022	0·0040
315	0·0028	0·0046	0·0022	0·0040	0·0020	0·0036
316	0·0024	0·0040	0·0022	0·0040	0·0024	0·0046
317	0·0028	0·0042	0·0022	0·0042	0·0020	0·0040
318	0·0024	0·0038	0·0024	0·0042	0·0020	0·0040
319	0·0026	0·0044	0·0022	0·0040	0·0020	0·0040
Variations	33%	>129%	27%	>117%	20%	>117%

Explanation of tables.—The tables have all been made according to the same principle. In Table I the cells of 12 rabbits (first column) have been used to determine the test doses of the three toxins (7, 17 and 19) for one unit of antitoxin. Thus it shows, for example, that when the cells of rabbit 314 are used, the test dose of toxin No. 7 for one unit of antitoxin amounts to 0·12 c.c. (no-hæmolysis endpoint) and 0·14 c.c. (complete-hæmolysis endpoint). In the same manner Table III shows that the test doses of toxin No. 17 for 0·01 unit of antitoxin amount to 0·0024 and 0·0040 c.c. respectively, when the cells of rabbit No. 313 are used, and to 0·0022 and 0·0036 c.c. respectively when the cells of rabbit No. 314 are used.

The results have been summarized in Table IV.

TABLE IV.—*Variations in Test Dose for* 1, 0·1 and 0·01 *Unit.*

Toxin.	1·0 Unit.		0·1 Unit.		0·01 Unit.	
	No hæmolysis.	Complete hæmolysis.	No hæmolysis.	Complete hæmolysis.	No hæmolysis.	Complete hæmolysis.
7	9%	33%	23%	75%	33%	>129%
17	20%	33%	17%	81%	27%	>117%
19	9%	14%	7%	61%	20%	>117%

This table shows that (*a*) the greatest variations occur in the test doses for 0·01 unit of antitoxin; the variations in test doses for 1 unit of antitoxin are the smallest; (*b*) the variations are much more considerable when the complete-hæmolysis endpoint is chosen than when the no-hæmolysis endpoint is determined; (*c*) this holds for all three toxins.

From this experiment it may be concluded that test doses differ when cells of different rabbits are used and that the differences are largest when the

complete-hæmolysis endpoint is read. It is therefore better to use the no-hæmolysis endpoint as the final reading, except when the cells of the same rabbit are always used. This condition can only be fulfilled in laboratories where these titrations are carried out very rarely. Even then the question arises whether the cells of the same rabbit always possess the same degree of resistance against hæmolysis. It must be noted that in the course of these experiments such variations have not come to light.

Titrations with Different Test Doses.

On account of the findings recorded above the following titrations were next carried out. For all experiments the cells of 4 rabbits were used (Nos. 295, 296, 297 and 312), whereas two toxins (Nos. 7 and 19) have been employed. Three sera were titrated, one sheep serum and two horse sera (one concentrated). These sera were suitably diluted for titrations with the test doses for 0·1 and 0·01 unit respectively. The last tube without a sign of hæmolysis was always taken as endpoint.

Example.—Table I shows that with the cells of the rabbits 295, 296 and 297 the test dose of toxin No. 7 was 0·11 c.c. ; with the cells of rabbit 312 it was 0·12 c.c. ; for toxin No. 19 the test doses were 0·11 c.c. (rabbit 295), and 0·12 c.c. (rabbits 296, 297 and 312). Now the undiluted sheep-serum (titre approximately 250 units) was titrated with the cells of all 4 rabbits and also with both test doses. This means that when toxin No. 7 was used and the cells of rabbit 295, 0·11 c.c. and 0·12 c.c. were taken as test dose, 0·11 c.c. being the correct test dose for these particular cells.

All titrations of this experiment are based on the same principle. The results are recorded in Tables V, VI and VII.

TABLE V.—*Titrations with Test Dose for* 1 *Unit of Antitoxin* (*Test Doses used :* 0·11 *and* 0·12 *c.c. for Toxins* 7 *and* 19).

Serum.	Titres.		Variation.	Titres when using correct test dose.		Variation.
	Lowest.	Highest.		Lowest.	Highest.	
Sheep	225	255	13%	240	255	9%
Horse	50	60	20%	55	60	9%
Horse, concentrated	220	255	16%	225	255	13%

TABLE VI.—*Titrations with Test Dose for* 0·1 *Unit of Antitoxin* (*Test Doses used :* 0·013 *and* 0·014 *c.c. for Toxin* 7 *and* 0·014 *and* 0·015 *c.c. for Toxin* 19).

Serum.	Titres.		Variation.	Titres when using correct test dose.		Variation.
	Lowest.	Highest.		Lowest.	Highest.	
Sheep	2·0	2·5	25%	2·0	2·3	15%
Horse	2·2	2·6	18%	2·2	2·5	14%
Horse, concentrated	2·2	2·6	18%	2·4	2·6	8%

TABLE VII.—*Titrations with Test Dose for* 0·01 *Unit of Antitoxin* (*Test doses used :* 0·0024–0·0028 *and* 0·0032 *c.c. for Toxin* 7, *and* 0·0022 *and* 0·0024 *c.c. for Toxin* 19).

Serum.	Titres.		Variation.	Titres when using correct test dose.		Variation.
	Lowest.	Highest.		Lowest.	Highest.	
Sheep	0·13	0·22	69%	0·16	0·21	31%
Horse	0·13	0·23	77%	0·17	0·23	35%
Horse, concentrated	0·17	0·24	41%	0·18	0·24	33%

Table V gives the results of the undiluted sera, titrated with test doses for one unit of antitoxin. The left half of the table gives the lowest and highest titres determined and the variations, calculated as percentages of the lowest titre. In this part of the experiment each serum has been titrated 16 times (viz. with the cells of 4 rabbits, two toxins and two test doses each). The right half gives the titres and variations when the titrations with the correct test doses were taken into consideration. It shows that for all three sera the variations are smaller in the latter case, viz. 9, 9 and 13 p.c. respectively, compared to 13, 20 and 16 p.c. in the former case.

The same holds for the titrations with test doses for 0·1 and 0·01 unit (Tables VI and VII). In Table VI each serum was titrated 16 times, as in Table V ; in Table VII 20 times, as three different test doses had been determined with toxin No. 7 (0·0024–0·0028 and 0·0032 c.c. *vide* Table III).

The experiment shows that the variations recorded are smallest when the correct test dose is used. This holds for titrations with test doses for 1, for 0·1 and for 0·01 unit of antitoxin. Especially in the last case the differences in accuracy are striking, viz. variations of 31, 35 and 33 p.c. compared to 69, 77 and 41 p.c. respectively (Table VII).

Tables V and VI also show that when titrations are made in full or in tenths units of antitoxin the variations remain well under 20 p.c. provided the correct test doses are used.

SUMMARY.

1. The test dose of a staphylococcus toxin, determined by the hæmolytic method, is influenced by the rabbit cells, used in the experiment.

2. This influence is especially striking when the first tube with complete hæmolysis is taken as an endpoint.

3. For this reason it is considered preferable to take the last tube in which no hæmolysis whatever takes place, as the endpoint.

4. Even then the degree of accuracy is higher when the correct test dose for the cells of one particular rabbit is used, especially when titrations in hundredths of units are required.

5. The best results are obtained when in a preliminary experiment the test dose of the toxin with the cells of one rabbit is determined. Cells of the same rabbit must then be used for the experiment proper.

REFERENCES.

GLENNY, A. T., AND STEVENS, M. F.—(1935) *J. Path. Bact.*, **40**, 201.
HARTLEY, P., AND LLEWELLYN SMITH, M.—(1936) *Quarterly Bulletin of the Health Organization of the League of Nations* (Sp. Nr.).
LLEWELLYN SMITH, M.—(1936) *J. Path. Bact.*, **42**, 227.
MCCLEAN, D.—(1937) *Ibid.*, **44**, 47.
MORGAN, F. G., AND GRAYDON, J. J.—(1936) *Ibid.*, **43**, 385.
PARISH, H. J., O'MEARA, R. A. Q., AND CLARK, W. H. M.—(1934) *Lancet*, **1**, 1054.

ANTIBACTERIAL IMMUNITY TO *STAPHYLOCOCCUS PYOGENES.*

CHAMP LYONS,

Assistant in Bacteriology and Surgery, Harvard Medical School.

From the Department of Bacteriology of the London Hospital and Medical College.

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THE staphylococcus has been isolated many times from the human blood-stream. The present-day conception of bacterial invasiveness as distinct from bacterial toxigenicity makes it unwise to assign only toxigenic power to an organism capable of creating a sustained bacteriæmia. Burnet (1929) has demonstrated that rabbits immunized with the *a*-lysin are protected from an acute death, but fail to clear intravenously injected staphylococci from the blood stream and may subsequently die with demonstrable antitoxin in the heart's blood. Smith (1937) has also found antitoxin in the blood of animals dying of staphylococcal infections. Downie (1937) has shown that antitoxic immunity modifies but does not prevent the skin lesion in rabbits. The results of these experiments with rabbits immunized to the *a*-lysin are not directly applicable to the treatment of human infections. The red cells and leucocytes of human blood are not susceptible to the toxic action of *a*-lysin, and leucocidin production appears to be more closely correlated with human pathogenicity (Panton and Valentine, 1932).

It is generally held that the virulence of invasive bacteria is dependent upon the capacity of the organisms to resist destruction in the body, and that an essential factor in protection is necessarily an antibacterial antibody. Evidence is presented here for an encapsulated invasive phase of staphylococcal growth and for the existence of a type-specific antibacterial antibody to the organism in this phase.

EXPERIMENTAL PROCEDURES.

Source of material.

The strains labelled A1 to A4 were obtained from direct platings of human pus and strains A5 to A8 were isolated in blood cultures. Strains labelled "S-" were obtained from Dr. S. T. Cowan of this laboratory. S11-R_3 was originally isolated from a septic arthritis and had been passed three times in rabbits. S80 was isolated from an osteomyelitic bacteriæmia and was used for Downie's (1937) experiments. S34 was a completely non-toxigenic strain recovered from normal human skin.

Aureus and *albus* variants were found to behave similarly in these experiments. There was no other evidence of colonial variation.

Cultivation.

Broth medium.—To filtered meat infusion is added Witte peptone (2 p.c.) and salt (1 p.c.). This is brought to pH 9·3 to precipitate phosphates and filtered. The pH is adjusted to 7·8 and glucose added to make a 0·05 p.c. solution. This is sterilized by steaming for 20 minutes on 3 successive days.

"Young" cultures are heavily seeded secondary cultures grown from 2 to 3 hours. An inoculum of 0·1 c.c. of overnight culture is used for each 5 c.c. of secondary culture. The broth is always heated to 95° C. and cooled before use. Growth is more abundant in shallow layer containers.

"Old" cultures are overnight cultures in the glucose broth made with the usual loop inoculum.

Demonstration of Capsules in Young Infusion Broth Cultures.

Hanging-drop preparations.—A drop of culture is mixed with one loopful of 15 p.c. colloidal silver ("collargol").

Capsule stain.—The smears are dried in the incubator for 30 minutes and never flamed. The slide is covered with undiluted carbol fuchsin and the excess decanted after 5 seconds, so that only a thin surface layer of stain remains, but this should not be allowed to dry. After 1 minute the slide is quickly rinsed in running water and covered with an aqueous solution of 2 p.c. potassium hydroxide for 10 seconds. This is then decanted and, without rinsing, Lœffler's alkaline methylene blue is added for 10 seconds. The slide is rinsed in water and blotted dry. The cocci stain blue and the capsules pink. The capsule is most readily seen when the smear is examined with a reduced light. The staining is usually best at the margins of the smear; it fades on keeping.

Demonstration of Capsules in Pus and Blood.

Capsule stain.—The slides are prepared and stained with carbol fuchsin as above. The slide is quickly rinsed in running water and then in "lysol" diluted 1/20 in distilled water. It is washed again in water and stained for 10 seconds with cresyl violet. The "lysol" rinsings decolorize the precipitated serum and must be adapted to the thickness of the smear. The cocci stain blue and the capsules pinkish purple.

Spontaneous Agglutination.

0·5 c.c of culture is added to 0·5 c.c. of saline in agglutination tubes and examined for agglutination after 3 hours in a 56° C. water bath.

Phagocytosis in Human Blood.

Source of blood.—The blood is obtained by aseptic venipuncture of healthy adults and defibrinated with glass beads in a glass bottle. Serum dilutions are made in saline. " Washed cells " are cells washed three times in saline. " Heated serum " is serum heated to 56° C. for 1 hour.

Technique.—The old cultures are diluted to a comparable turbidity with the young cultures by adding 3–5 c.c. of fresh broth to 1 c.c. of overnight culture. Pasteur pipettes are adjusted to equal bore by breaking them at the point where the outside diameter just occludes a 48-gauge drill hole in a metal strip. One drop of culture is added to 0·25 c.c. of blood in a pyrex tube. The tube is sealed and placed in a 37° C. water-bath for 5 minutes and then in a rotating box in a 37° C. incubator for 15 minutes. The tube is then flamed, broken open and a blood film made and stained with Wright's stain. Counts are made of the number of intracellular cocci seen in 50 consecutive polymorphonuclear leucocytes and the percentage of cells taking part in the phagocytosis is noted. Complete details of this technique are given by Ward and Enders (1933).

Bactericidal Tests in Human Blood.

The modified Todd technique described by Ward (1930) has been used. Serial ten-fold dilutions of culture are made in plain broth. Agar plate surface counts are made in duplicate of the number of bacteria in one drop of the two lowest dilutions. One drop of each dilution is added to 0·25 c.c. of blood as for the phagocytic test and the tubes rotated for 20 hours in the incubator. After rotation the contents of each tube are cultured in 5 c.c. broth to determine the presence of viable cocci.

Leucocyte Counts.

2 c.c. of overnight culture are centrifuged and resuspended in 1 c.c. of fresh broth. 1 drop of this is added to a pyrex tube and mixed for 5 minutes with 1 drop of the serum to be tested. The toxicity of foreign serum for human leucocytes is such that rabbit serum should be used diluted 1/10 and horse serum 1/5. 0·5 c.c. of defibrinated human blood is added to each tube and the tubes are sealed and rotated as in the phagocytic test for 2 hours. White cell counts are made immediately after adding the blood and after rotation. Blood samples are taken in Thoma pipettes, diluted in 1 p.c. acetic acid and mechanically shaken for 30 minutes to break up any clumps. Counts are made in the " improved Neubauer " chambers in accordance with standard practice.

Slide Agglutination.

The technique employed has been that adapted by Griffith (1935) for hæmolytic streptococci and readings have been made in accordance with the photographic chart published by Pauli and Coburn (1937). Young culture suspensions are prepared by placing a culture in the water bath at 80° C. and slowly heating to 100° C. This heat-killed culture is centrifuged and resuspended in 0·25 c.c. of plain broth for each 10 c.c. of original culture. Old culture suspensions need not be heated and are resuspended after centrifuging in 0·25 c.c. of plain broth for each 5 c.c. of original volume. The test mixture is 1 drop of suspension and 1 loopful of serum diluted 1/5 in saline. Final readings are taken after 5 minutes, Cultures for agglutination should not be made directly from stock, *i. e.* cultures which have had daily serial passages in broth for 3–4 days are necessary for specific reactions.

Antitoxin Titrations.

The anti-α-lysin values were determined against standardized toxin with a 2 p.c. suspension of rabbit red cells in saline as indicator.

Blood Clearance Experiments.

3 c.c. of overnight culture are centrifuged and resuspended in 1 c.c. of fresh broth for a single infecting dose. The rabbits are allowed water on the morning of the experiment. Both ears are shaved, washed with soap and water and dried with alcohol. Injections are made in a marginal ear vein and bleedings are made from stab wounds in the marginal vein of the opposite ear. The

first sample is taken from the base of the ear and subsequent stabs are always distal to the previous wound. Broth with 0·4 p.c. citrate is used to dilute the blood collected in calibrated pipettes. 0·5 c.c. of blood is mixed with 1 c.c. of citrate broth in a Petri dish and 0·2 c.c. of blood is diluted in 1·8 c.c. of citrate broth to obtain a 10^{-1} dilution. Subsequent dilutions to 10^{-3} are made with 0·5 c.c. in 4·5 c.c. of plain broth. A single pour plate is made of the 0·5 c.c. of blood and duplicate pour plates are made with 0·5 c.c. of each of the dilutions. The plates are incubated 24–36 hours and the bacteria per c.c. of blood estimated from the colony counts. Animals surviving one week are sacrificed for autopsy and cultures.

EXPERIMENTAL RESULTS.

The Encapsulation of Staphylococci.

The microscopical examination of hanging drop preparations and stained smears from young infusion broth cultures of toxigenic and non-toxigenic strains of staphylococci shows the bacteria to be essentially encapsulated diplococci. Tetrads, triads and single forms are also present, but these are regarded as intermediate forms in the reproduction of the diplococcus. The familiar " clusters " do not occur, encapsulation is uniform and the cocci are large. The capsules are not demonstrable after agitation on a mechanical shaker, but withstand heating to 100° C. for 5 minutes. The old infusion broth cultures contain a few encapsulated and many non-encapsulated bacteria. The encapsulated forms are like those seen in the young culture and the non-encapsulated organisms are small and clumped to form the well known " clusters ".

Similar studies have been made with old cultures of toxigenic strains planted in freshly drawn defibrinated human blood and incubated at 37° C. in a rotating machine. The optimal inoculum is 1000–10,000 cocci in 0·25 c.c. of blood. During the active growth phase (3–8 hours) cocci occur inside and outside the leucocytes. The extracellular organisms are the large encapsulated diplococci and the intracellular cocci are both large and small. Later smears (20–28 hours) demonstrate a great reduction in the number of polymorphonuclear leucocytes with a preponderance of free cocci in the clumped small non-encapsulated form. If a million or more cocci are added to the same amount of blood the 3–8-hour smears resemble the later smears from the blood with the small inoculum.

Culture in infants' or adult human serum for 20 hours constantly produces a flocculent growth of organisms which are spontaneously agglutinable in saline. Infusion broth cultures grow diffusely and do not agglutinate in saline. Young serum-grown organisms are not encapsulated and occur in clusters. Table I summarizes a series of cultural experiments which emphasize this effect of serum. Serial passage in serum was found not to modify subsequent encapsulation in infusion broth or defibrinated blood.

Non-toxigenic strains which produced capsules in young infusion broth cultures did not maintain these capsules in blood and the organisms were phagocyted and killed in large numbers in the bactericidal test. Three facts are apparent: First, the toxigenic strains do not produce capsules in serum but do encapsulate in blood; second, both toxigenic and non-toxigenic strains are capable of encapsulation, but only the toxigenic strains can maintain their capsules in blood; and third, although both toxigenic and non-toxigenic

TABLE I.—*The Influence of Serum upon the Encapsulation of Pathogenic Staphylococci.*

Culture medium.	Capsules in 3-hour cultures.	Growth at 20 hours.	Spontaneous agglutination of 20-hour cultures.
0·05 p.c. glucose infusion broth	+	Diffuse	0
1 p.c. peptic digest blood in infusion broth	+	,,	0
1 p.c. laked washed red cells in infusion broth	+	,,	0
1 p.c. laked whole blood in infusion broth	0	Flocculent	+
1 p.c. unheated serum in infusion broth	0	,,	+
1 p.c. heated serum in infusion broth	0	,,	+
Defibrinated human blood	+	..	..

strains are phagocyted, only the non-toxigenic strains are killed by the blood. In order to explain these observations it is suggested that both strains lose their capsules in the serum of defibrinated blood and are then phagocyted by the leucocytes, but the toxigenic strain is able to reproduce its encapsulated phase therein and to return to the serum by destruction of the leucocyte. A destruction of the leucocytes by toxigenic strains can be shown to occur by adding approximately ten million organisms to 0·5 c.c. of defibrinated blood and incubating for 2 hours at 37° C. in the rotating machine (see Table II).

TABLE II.—*The Bactericidal Power of Blood for Toxigenic and Non-toxigenic Staphylococci.*

Strain.	Leucocyte counts (per c.mm.). Before rotation.	Leucocyte counts (per c.mm.). After rotating 2 hours.	Bacteria killed by 0·25 c.c. blood.
Toxigenic A5	4550	2350	4
Non-toxigenic S34	4450	4500	300,000

The Phagocytosis of Staphylococci.

The old infusion broth cultures and the serum-grown cultures are so susceptible to phagocytosis that 15 minutes' exposure to the blood suffices to demonstrate large numbers of intracellular cocci. Young infusion broth cultures are phagocyted only in small numbers during the first 15 minutes' exposure to the blood, but thereafter the number of intracellular cocci slowly increases. It requires 60 minutes, however, for the phagocytic values to equal those reached in 15 minutes by the old infusion broth or serum cultures. The 15-minute period was therefore selected as the optimum for the purpose of the test. In Table III is presented an experiment which demonstrates that

TABLE III.—*The Phagocytosis of Young and Old Cultures of Toxigenic Strains.*

NOTE.—The phagocytic count is expressed as 196–34% to indicate that 34% of 50 consecutive polymorphonuclear leucocytes contained a total of 196 intracellular cocci.

Test mixtures.	Strain S11 R_3.		Strain A5.	
	Young.	Old.	Young.	Old.
Washed cells + unheated serum	196–34%	526–96%	44–16%	192–86%
Washed cells + heated serum	66–10%	198–40%	24–8%	58–30%
Washed cells + unheated serum diluted 1 : 8 in saline	34–4%	221–42%	23–16%	44–26%
Washed cells + heated serum diluted 1 : 8 in saline	0–0%	62–14%	0–0%	26–12%
Washed cells + saline	0–0%	18–10%	0–0%	14–6%

the encapsulated young culture is more resistant to phagocytosis than the non-encapsulated old culture; the destruction of complement by heat retards the phagocytic process; the dilution of serum impairs phagocytic efficiency; and spontaneous phagocytosis is responsible for a negligible amount of the total phagocytosis. The phagocytic efficiency of whole blood is here clearly dependent upon a factor in the serum. Such non-specific phagocytosis in human blood has been demonstrated with old cultures of pneumococci (Ward and Enders, 1933) and hæmolytic streptococci (Ward and Lyons, 1935), but is not followed by the intracellular digestion of the cocci. It is believed that the delayed phagocytosis of the young culture is attributable to this same serum factor acting upon the somatic structure of the cocci after the capsule has been lost as a result of growth in the serum component of defibrinated blood.

The capsule seems to be the essential mechanism for resistance to phagocytosis because non-toxigenic strains also show a temporary resistance to phagocytosis in the encapsulated phase. Young cultures of toxigenic strains are still resistant after heating to 100° C. for 10 minutes to destroy the toxin (Burnet, 1929, 1931) and kill the culture. Antitoxin does not enhance phagocytosis. However, the addition of a toxin containing both *a*-lysin and leucocidin to the blood depresses the phagocytosis of the young culture. This is thought to be a toxic effect upon the leucocytes since the same result is obtained with blood which has been reconstituted after washing the cells and exposing them for short periods to low dilutions of the toxin.

These experiments have been presented to demonstrate that the encapsulated organisms are resistant to phagocytosis. It has been shown that the capsule does not persist in serum and that the young culture is ultimately phagocyted in consequence of non-specific sensitization by a serum factor capable of acting upon the cocci only after the capsule has disappeared. This secondary phagocytosis is bactericidal for non-toxigenic strains, but does not enable the leucocytes to destroy toxigenic organisms. The "opsonic index" as determined with old cultures is thereby invalidated as a test of antibacterial immunity to pathogenic staphylococci. The effects obtained by specifically sensitizing encapsulated organisms with anti-capsular antibody remain to be discussed.

Slide Agglutination of Staphylococci.

The agglutination reactions of young infusion broth cultures have been found to be the same with living organism and with cocci killed by heating to 100° C. Living cultures continue to grow out at room temperature so that it is necessary to use heat-killed organisms for constant results.

Eight rabbits were immunized with strain A5 ; Nos. 189 and 200 received 3-hour culture killed by heating to 80° C. for 5 minutes ; Nos. 191 and 201, 3-hour culture killed by heating from 80° C. to 100° C. ; Nos. 202 and 203, overnight culture killed by heating from 80° C. to 100° C. The vaccines were freshly prepared for each injection and were not washed or resuspended. Each rabbit received approximately 8·0 c.c. in doses of 1·0 c.c. of dead culture intravenously during five weeks. The overnight cultures contained many more organisms than the young cultures. Trial bleedings were made a week after the last injections and Table IV summarizes the agglutination reactions given by these sera.

TABLE IV.—*Slide Agglutination of Young and Old Cultures.*

	Serum diluted 1 : 5.	Strain A5.		Strain S11 R_3.	
		Young.	Old.	Young.	Old.
	Normal	0	0	0	0
Immunized with young culture of A5	189	+++	++++	0	++
	200	++	++++	0	+
	191	+++	++++	0	++
	201	+++	+++	0	++
Immunized with old culture of A5	202	0	++++	0	++++
	203	+	++	0	++
	204	+	++++	0	++++
	205	0	++++	0	++++

The agglutination of the young cultures appears to be type-specific ; that of old cultures largely a group reaction. The rabbits immunized with young cultures yielded sera containing relatively more type-specific agglutinin, whereas the sera from the rabbits immunized with the overnight cultures possessed more group agglutinin. Normal rabbit serum did not possess any agglutinins. Prolonged immunization of rabbits Nos. 203 and 204 with overnight cultures yielded sera with type-specific agglutinins as well as group agglutinins. Repeated attempts to demonstrate a " Quellung " phenomenon have been unsuccessful.

The Antibacterial Effects of the Antibody to the Capsules.

In Table V is recorded an experiment which demonstrates three in-vitro tests for the anticapsular antibody.

Encapsulated organisms were concentrated 10 times in 1/10 dilutions in

TABLE V.—*In-vitro Tests for the Anticapsular Antibody.*

Young culture.	Agglutination.	Phagocytosis.	Leucocyte counts (per c.mm.). Before rotation.	After rotating 2 hours.
A5 concentrated 10 times in 1 : 10 normal rabbit serum.	0	364–92%	6500	2100
A5 concentrated 10 times in 1 : 10 immune rabbit serum.	++++	785–98%	6350	6200
A5 broth culture not concentrated	..	38–20%	..	..

broth of normal and immune sera. At the end of 5 minutes agglutination was visible in the immune serum preparation and the test mixtures were then added to blood for the phagocytic test. The number of intracellular cocci in the blood which received the culture concentrated 10 times in normal serum is essentially a tenfold multiple of the number of organisms phagocyted by the blood which received the unconcentrated culture in broth. This relationship suggests that the basic phagocytosis observed at the end of 15 minutes with the young culture is due to the action of the non-specific opsonin and is independent of the capsular antibody. The enhanced phagocytosis of the organisms in the immune serum preparation is attributed chiefly to sensitization of the capsule with specific antibody. The leucocyte counts were made with the parent cultures of the organisms used in the phagocytic experiment. At the end of 2 hours there was no destruction of the leucocytes by the cocci which had been exposed to the immune serum containing anticapsular antibody This indicates that the older cultures can be specifically sensitized and that such sensitization enables the leucocytes to deal with the ingested organisms more effectively.

The current controversy as to the therapeutic value of antitoxin in the treatment of staphylococcus bacteriæmia prompted the experiments given in Table VI. A sample of concentrated antitoxin prepared by Dolman was found to contain specific agglutinins for strain S80, but not for strain A6. The leucocyte test indicates that this antitoxin has an effective antibacterial action for the strain which it agglutinates, and that this action is confined to that agglutinative type. Further evidence that this is due to a strict antibacterial antibody is supplied by the failure of a non-agglutinating homologous rabbit antitoxin similarly to prevent the destruction of the leucocytes. This necessitates a revised interpretation of the clinical results of therapy heretofore regarded as purely antitoxic in its action.

It was hoped that the bactericidal test would demonstrate the effective antibacterial action of the type-specific antibody, but no really significant killing was obtained. This failure has not been considered to discredit the specific antibody, because the toxicity of foreign serum for human leucocytes prevented the addition of sufficient of this relatively weak serum for an effective test ; therefore the ability of normal and immune rabbits to sterilize the blood-stream following the intravenous injection of living organisms was investigated.

TABLE VI.—*The Demonstration of Antibacterial Antibody in a Therapeutic Antitoxin.*

Experiments.	Added to 0·5 c.c. blood.	Leucocyte counts (per c.mm.). Before rotation.	Leucocyte counts (per c.mm.). After rotating 2 hours.	Slide agglutination of young cultures.
I	S80 + saline	6900	3900	0
	S80 + Dolman's antitoxin 1 : 5 (horse)	6700	6550	++++
	S80 + homologous antitoxin 1 : 10 (rabbit)	6800	4200	0
II	S80 + saline	5700	3800	0
	S80 + Dolman's antitoxin antitoxin 1 : 5	5650	5750	++++
	A6 + saline	6000	3950	0
	A6 + Dolman's antitoxin 1 : 5	6100	4150	0

Table VII summarizes the results of such blood clearance experiments performed with normal and immunized rabbits. The term "immune" is used to designate those rabbits which possessed type-specific agglutinins for encapsulated organisms in their sera after immunization. It is believed that the courses of these infections are a corroboration *in vivo* of the results of the phagocytic experiments; namely, that two phagocytic factors exist for the staphylococcus. In all animals there is a considerable initial clearance of organisms during the first hour after infection, but only those animals whose sera contain the specific agglutinin are capable of further reduction of the bacteriæmic counts. In the normal rabbits the opsonizing factor is non-specific and ineffective, as evidenced by the subsequent steady increase in the number of circulating organisms. In the immune rabbits the type-specific antibody sensitizes the bacteria for intracellular digestion and leads to a progressive diminution of the staphylococci in the blood. The type-specificity of this antibody is again demonstrated by the failure of an immunized animal to clear the agglutinatively heterologous S11 R_3 from the blood. The agglutinatively homologous strain S80 is highly toxigenic and the rabbit possessing only antibacterial immunity was unable to maintain the reduced intensity of the bacteriæmia apparent during the first 7 hours. The depression of the phagocytosis of encapsulated cocci by a toxin containing α-lysin and leucocidin has been discussed. Burnet (1929) has found that rabbits immunized with the α-lysin alone are unable to clear the blood in similar experiments, but that antitoxic immunity does protect against the secondary rise of the bacteriæmia which occurs after eight hours. The experiments reported here demonstrate that the antibacterial antibody leads to an effective blood clearance which may permit the survival of the animal even in the absence of antitoxic immunity. However, the importance of antitoxin in staphylococcal immunity is fully recognized and it would appear that therapeutic sera for the treatment of an established staphylococcal infection should contain both antibacterial and antitoxic antibodies.

TABLE VII.—*The Clearance of Intravenously Injected Staphylococci from the Blood Stream of Normal and Immunized Rabbits.*

Experiments.	Rabbits.	Units anti-α-lysin per c.c. serum.	Strain injected.	Bacteria per c.c. of blood. 5 mins.	1 hr.	3 hrs.	5 hrs.	7 hrs.	24 hrs.	Result.	Staphylococci at autopsy. Heart's blood.	Kidney.
I	Normal	2	A5	183,000	730	90	110	Not done	*	d., 6 days	0	+
	Immune No. 204	1	A5	180,000	204	54	4	„ „	*	d., 5 days	0	+
II	Normal	<0·1	A5	61,000	460	220	310	420	970	d., 3 days	+	+
	Immune No. 205	2	A5	47,000	144	108	14	10	Sterile	S.	0	+
III	Normal	<0·1	A5	320,000	1050	250	500	3730	—	d., 18 hrs.	+	+
	Immune No. 189	<0·1	A5	201,000	210	46	256	280	390	d., 4 days	0	+
	„ No. 191	<0·1	A5	95,000	250	50	30	80	Sterile	S.	0	+
IV	Normal	<0·1	S80	203,000	250	80	50	140	108	d., 5 days	+	+
	Immune No. 201	<0·1	S80	93,000	130	34	16	28	120	S.	0	+
V	Normal	<0·1	S11 R_3	143,000	340	100	70	110	22	d., 3 days	+	+
	Immune No. 200	<0·1	S11 R_3	120,000	310	142	60	100	36	S.	0	+

All immune rabbits were prepared with heat-killed cultures of strain A5. Strains A5 and S80 are agglutinatively homologous and S11 R_3 is heterologous.

* = Spontaneous infection with a motile, late lactose-fermenting Gram-negative bacillus. d. = Death of the animal. S. = Survived 7 days, but sacrificed for autopsy.

COMMENT.

It has been found possible to demonstrate the capsule of the staphylococcus. This capsule is developed by toxigenic and non-toxigenic strains in young (3-hour) infusion broth cultures and has largely disappeared in older (20-hour) cultures. Growth in serum inhibits encapsulation by some action not understood at the present time. The capsule resists heating to 100° C. for five minutes but can be removed by mechanical agitation. The toxigenic strains are able to reproduce the encapsulated phase and grow out in defibrinated human blood, but the non-toxigenic strains are killed by the blood. Both toxigenic and non-toxigenic cocci in the encapsulated phase are resistant to phagocytosis but growth in the serum component of defibrinated blood induces a non-encapsulated phase which is susceptible to phagocytosis. Experiments are quoted which indicate that this phagocytosis is effected by opsonization of an antigenic complex distinct from the capsular antigen by a phagocytic factor usually present in human serum. This non-specific phagocytosis is bactericidal for non-toxigenic strains, but leads to the destruction of the leucocytes with the toxigenic strains. Panton and Valentine (1932) have demonstrated that leucocidin is the only known staphylococcal toxin which is effective against the human leucocyte and have correlated leucocidin production with bacterial invasiveness in human infections. It is suggested that leucocidin inhibits the intracellular digestion of toxigenic staphylococci which have been phagocyted in consequence of non-specific sensitization. The microscopical evidence indicates that the toxigenic strains phagocyted in the non-encapsulated phase reproduce their capsule within the leucocyte where they are protected from the serum. The ultimate destruction of the leucocyte permits the cocci to return to the blood stream in the encapsulated and invasive phase. Antitoxin cannot be expected to neutralize this intracellular toxin and does not prevent the destruction of leucocytes by toxigenic strains in direct experiment.

Slide agglutination of heat-killed encapsulated cultures is the most satisfactory method of identifying anticapsular antibody. Rabbits immunized with heat-killed young cultures yield sera containing type-specific agglutinins, and such sera demonstrate that the surface antigen of the staphylococcus in the encapsulated phase is different from the surface antigenic complex of the non-encapsulated phase. Sera which contain agglutinins for the encapsulated organisms also specifically and effectively sensitize encapsulated organisms for phagocytosis and prevent the destruction of leucocytes by toxigenic strains. Rabbits which contain such agglutinins in their sera are enabled to clear their blood streams of intravenously injected staphylococci more efficiently than normal rabbits. In other words, the anticapsular antibody appears to be the essential antibacterial antibody for the staphylococcus. It is further indicated that some of the therapeutically successful antitoxins may owe their usefulness in part to this antibacterial antibody.

CONCLUSIONS.

1. Staphylococci are encapsulated in the invasive phase.
2. The effective antibacterial antibody is a type-specific antibody to the capsular antigen.

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REFERENCES.

BURNET, F. M.—(1929) *J. Path Bact.*, **32**, 717.—(1931) *Ibid.*, **34**, 759.
DOWNIE, A. W.—(1937) *J. Path. Bact.*, **44**, 573.
GRIFFITH, F.—(1935) *J. Hyg.*, Camb., **34**, 542.
PANTON, P. N., AND VALENTINE, F. C. O.—(1932) *Lancet*, **1**, 506.
PAULI, R. H., AND COBURN, A. F.—(1937) *J. exp. Med.*, **65**, 595.
SMITH, M. L.—(1937) *J. Path. Bact.*, **45**, 305.
WARD, H. K.—(1930) *J. exp. Med.*, **51**, 675.
Idem AND ENDERS, J. F.—(1933) *Ibid.*, **57**, 527.
Idem AND LYONS, C.—(1935) *Ibid.*, **61**, 515 and 531.